Effect of foreign (=not self) DNA/RNA on the Human Immune System in regard to Genetically Modified Plants

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This review shall give policy makers and risk managers the rational basis to widen the view on the risk assessment of genetically modified plants and therefore to include potential impacts associated with synthetic DNA/RNA fragments of genetically modified plants to the human immune system. Such risks are so far excluded form the risk assessment of genetically modified plants carried out by the European Food Safety Authority (EFSA). The EC Commission bases all approvals on the conclusion in the risk assessment of EFSA

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Zusammenfassung:

Man unterscheidet zwei Arten von Immunsystem. Das angeborene und das adaptive Immunsystem. Das angeborene Immunsystem orientiert sich bei der Erkennung von Mikroorganismen und Viren an alte, universell gültige evolutionär konservierte molekulare Muster. Zu diesen zählen auch DNA und RNA, die offensichtlich stärker konserviert und sich weniger schnell im Laufe der Anpassung der Organismen verändern als andere Zellbestandteile der Mikroorganismen.oder Viren. Im Menschen sind mehrere Muster erkennende Rezeptoren aktiv, die meisten gehöen zu der Famile der Toll-like-Rezeptoren (TLR). Von diesen Rezeptoren erkennen TLR3 doppelsträngige RNA und mRNA, TLR7 und TLR8 dopelstängige DNA, sowie TLR9 CpG DNA. Daneben gibt es noch TLR unabahängige Rezeptoren die ebenfalls DNA Moleküle erkennen. Gentechniker erstellen synthetischen Genabschnitte in transgenen Pflanzen ohne die alten, universell gültigen evolutionär konservierten molekularen Muster zu kennen. Fragmente von Nahrungs DNA überstehen die Verdaung und können im Blut und Lymphsystem sowie mehreren Organen wie Leber, Milz, Niere nachgewiesen werden. Dies wurde auch für synthetische DNA Fragmente aus gentechnisch veränderten Pflanzen nachgewiesen. Für bakteriellere Nahrungs DNA konnte nachgewiesen werden, dass in jenen Geweben wo Nahrungs-DNA gefunden werden konnte, mit der Aktivität dieser Nahrungs DNA korrellierte Unter diesem Gesichtspunkt dürfte die Präsenz von Nahrungs DNA in den Geweben nicht rein zufällig sein. Eine immunmodulatorische Funktion von Nahrungs-DNA aus Pflanzen ist als sehr wahrscheinlich anzusehen. Da synthetische Nahrungs-DNA aus gentechnisch veränderten Pflanzen synthetisch hergestellt worden ist, und sich von natülicher DNA Sequenzen der konventionell gezüchteten Pflanzen unterscheidet, dürfte die immunologische Wirkung von synthetischer DNA aus gentechnisch veränderten Pflanzen von natürlicher DNA-Sequenzen verschieden sein. Die Wirkung synthetischer DNA Fragmente auf das Immunsystem und Organe des Menschen wird aus der amtlichen Risikoabschätzung durch die Europäische Behörde für Lebensmittelsicherheit (EFSA) ausgeblendet. Ein Forschungsschwerpunkt, der sich der Wirkung von synthetischen DNA und RNA Fragmenten aus transgenen Pflanzen widmet ist dringend notwendig um zu klären, ob diese synthetischen DNA Fragmente eine Gesundheitsgefährdung für den Menschen darstellen oder

nicht. Ohne Analyse der Wirkungen von synthetischen DNA Sequenzen auf das Immunsystem des Menschen ist eine abschließende Bewertung über die Sicherheit der gentechnisch veränderten Pflanzen nicht möglich.

Abstract

There are two different forms of the immune system in humans. The innate and the adaptive immune system. The innate immune system recognizes universal, evolutionary conserved patterns so called pathogen associated patterns (PAMP) via pattern recognition receptors (PRR) and is the so called "first line of defense". DNA and RNA sequences are PAMPs which do have immuno-modulatory functions. Many PRR belong to the Toll-like-receptor (TLR) family. Where TLR 3 recognizes double stranded RNA, TLR7 and TLR8 recognize single-stranded RNA and TLR9 is a receptor for CpG DNA. Besides that there are TLR independent receptors which do also recognize DNA and RNA. Genetically modified plants carry man made synthetic genes (DNA sequences) which do not occur in any living species. Scientists do produce genetically modified plants but do not understand old and universal patterns of DNA sequences which are recognized by the immune system. Fragments of food DNA and fragments of synthetic sequences are not fully degraded during digestion but can be detected in the lymph system, the blood, and several organs like liver, spleen muscles. For food DNA from bacteria it was detected that the location of the food DNA coincided with the immunomodulatory activity of this bacterial food DNA. In this light the presence of fragments of synthetic DNA sequences from genetically modified plants in the blood, liver etc. is very likely to coincide with yet unknown immunomodulatory activity. As genetically modified plants contain synthetic DNA sequences which are new to the immune system the type of immunomodulatory activity might be quite different to those evolutionary evolved "natural food DNA sequences". The European Food Safety Authority (EFSA) was and is still very silent on this issue. So far immunomodulatory activity of synthetic DNA sequences from genetically modified plants have been excluded from the risk assessment. An exploratory focus (or a research program) is urgently needed to analyze the immunomodulatory activity of synthetic DNA sequences from genetically modified plants. The safety of genetically modified plants on human health cannot be determined, unless these urgent questions have been clarified.

1 INTRODUCTION

The genome and the cell are hardly understood, as following citations show:

> *" We really have a poor understanding of what a gene actually does and where and when it should do it. You can understand the entire genome and [still] understand less than 1 percent about what is going on in a cell." Eric Neumann, vice president of bioinformatics at Beyond Genomics Inc. (DODGE 2003).*
>
> *"We are in a data-rich environment, but the fact is we are information-poor. You look at biological systems with much more complexity than before." Peter Sorger, an associate professor of biology at MIT (DODGE 2003)*

Fundamental gaps of knowledge in the understanding on gene interactions point at gaps in the risk assessment of genetically modified plants. Besides knowledge gaps in molecular biology there are also knowledge gaps on the interaction of genetically modified plants with the human immune system and ecosystem.

The current practice of risk assessment of human health effects of genetically modified plants by the European Food Safety Authority (EFSA) is mainly based on the "Concept of Substantial Equivalence" and on the evaluation of risk associated with the novel protein. The limitations of this narrow scientific approach in the risk assessment of genetically modified plants has been questioned by several authors (MILLSTONE et al. 1999, PUSZTAI et al. 2003, MÜLLER 2002, MÜLLER 2004, MUELLER et al. 1999, SPÖK et al. 2002, MILLSTONE 2002, SPÖK et al. 2003). Up to now EFSA has refused to address potential risks associated with the synthetic nature of the DNA and RNA of transgenic plants on human health. This report shall give an overview on the current state of knowledge on interactions and pathways of external DNA with the immune system.

2 SYNTHETIC GENES

2.1 Introduction

In the discussion on uncertainties and precaution associated with the uptake of synthetic DNA from food, the question arises whether there is a difference between genes inserted by conventional breeding methods and synthetic transgenes inserted by gene technology. Many scientists (ILSI 2002) assume that there is no difference between these two types of genes and therefore risks of synthetic DNA or RNA uptake has to be compared with risks of DNA from conventionally bred crops. The following chapters give a short overview of differences between genes and synthetic transgenes.

2.2 Structural difference of genes and synthetic transgenes

1. Synthetic genes are a new combination of various genetic elements from different organisms.
2. As plants do not well interpret some sequences taken from bacterial genome. The sequences taken from different organisms are afterwards manipulated (truncated, exchange of sequences) to reach a certain level of expression.
3. Thus getting a completely synthetic sequence which does not occur in any living species on the planet.

See gives an overview on the different genetic elements (DNA sequences) from different organisms.

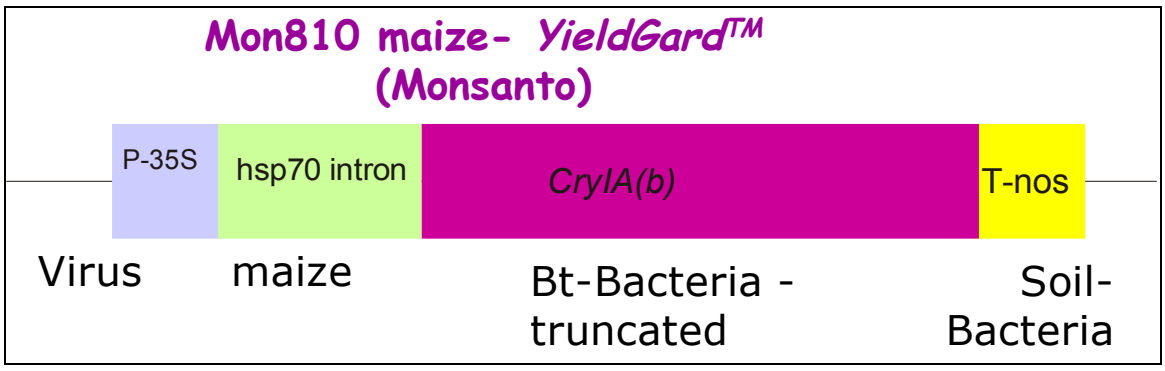

Figure 1: Synthetic gene used in the insect resistant transgenic plant Mon 810. The synthetic gene consists of 4 different genetic elements from 4 different organisms. Sequences of these 4 genetic elements are adapted ie.

exchange of base pairs. The result is a completely synthetic sequence which does not occur in any living species on earth.

The following citation from a company gives a good picture why there is a need to synthesize transgenes and to produce genes which do not occur in the nature but have to be designed on a rational basis.

> *"Parameters such as codon usage, GC content, cryptic splice sites, premature poly(A) sites, AT rich killer sequences, RNA secondary structures, and host sequence identities (RNA interference), frequently limit heterologous and autologous gene expression in plants down to undetectable levels of the gene product. This dilemma often makes it necessary to adapt and optimize the gene of interest towards the genetic requirements of the host organism.* ***Apparently, an optimized sequence does not occur in nature and has to be designed on a rational basis followed by in vitro synthesis****.*
> *GENEART offers both steps:* ***state-of-the-art gene optimization plus fast and reliable de novo gene synthesis****.*
> *GENEART helps you to* ***fully control every feature of your gene****:* ***Adapt*** *codon usage and GC content for optimal translation efficiency.* ***Eliminate*** *premature poly(A) sites, cryptic splice sites, killer sequences and RNA secondary structures to increase the level of full length mRNA.* ***Avoid*** *homologies to host genes to prevent gene silencing through RNA interference.* ***Increase*** *genetic stability in transfer organisms such as E. coli and A. tumefaciens****. Include and exclude*** *restriction sites.*
> *GENEART's proprietary, patent pending* ***gene optimizing software GeneOptimizerTM*** *allows for the simultaneous* ***adaptation*** *of all these parameters, together with additional requirements defined by the scientist. It identifies the single best sequence among an infinite number of possible combinations coding for a given protein. Highly automated* ***de novo gene synthesis*** *at GENEART ensures the most cost-effective, fast and*

accurate production of virtually any DNA sequence with very short delivery times.

http://www.geneart.com/fileadmin/user_upload/pdfs/transgenic_plants.pdf

2.3 Difference of genes and synthetic transgenes on the plant genome

1. One mayor difference is that normal plant breeding do not touch the gene and do not influence the chromosomes. Many species do have a double (or a sixfold) set of chromosomes. Which is like a backup copy of every gene.. In normal plant breeding each gene has thus a corresponding gene on the neighbor (corresponding) chromosome (at diploid, tetraploid, hexaploid species). Transgenes are shot by a gene gun into the genome. The integration of the synthetic gene occurs randomly anywhere on the genome in any of the chromosomes. There is no corresponding gene on the neighbor chromosome.
2. It is not possible to locate the synthetic transgene at special sites of the plant DNA. While the functions of all sequences the crop plant genome (genes and other non-coding regulatory elements) is unknown, the integration may occur on sites of the DNA with regulatory functions. Disruptions in the expression of protein, introns or other non-coding RNA-genes are highly likely. While the whole genome of the crop is not understood, most of these disruptions will remain undetected.
3. In most of the cases gene insertion goes hand in hand with deletion of parts of the plant genome. In many cases small deletions (1-100 base pairs) are associated with the insertion of a synthetic gene. But many of the important genetically modified plants do have also very large deletions. E.g a deletion of at least 12.000 base pairs or rearrangement may have occurred in the Round up Ready Soybean (WINDELS et al. 2001). 10 years after the approval it is still not known what has happened with the genome after the insertion of the synthetic transgene. The largest reported deletion on the crop plant genome by insertion of a synthetic transgene was 78.000 base pairs (removing 13 genes). (Kaya et al 2000 cit. In WILSON et al. 2004).
4. After the insertion of the synthetic transgene, rearrangements of the synthetic transgene and/or neighbored sequences occur in the plant. In any cases, the synthetic transgene in the plant is not the same as inserted into

the plant. Many parts of the synthetic transgene are truncated or in other forms changed by the plant's own mechanisms.(e.g. maize: Mon810, Bt176, GA21) (COLLONIER et al. 2003).

5. New unintended sequences which neither belong to the insert nor belong to the crop plant DNA are found the crop plant genome adjacent to the place of insertion (COLLONIER et al. 2003, WINDELS et al. 2001). These sequences produce RNA but not proteins e.g. Roundup-ready soybean (RANG et al. 2005) and maize NK603 (EFSA 2003). The functions of these new (synthetic) RNAs are not known.

6. Every insertion of synthetic transgene is associated with thousands of mutations on the plant genome. These mutations can be eliminated by several (conventional) back crosses (WILSON et al. 2004).

7. The synthetic transgene integration changes the transcription pattern and methylation of the genome. This is not only associated with natural sequences of the plant nearby the integration site of the synthetic transgene, but also with sequences on further distances (DOERFLER et al. 2001a). Figure 2 gives an overview on the differences between the synthetic gene before insertion into the plant and after insertion detected in the plant by sequence analyses undertaken by independent research institutions.

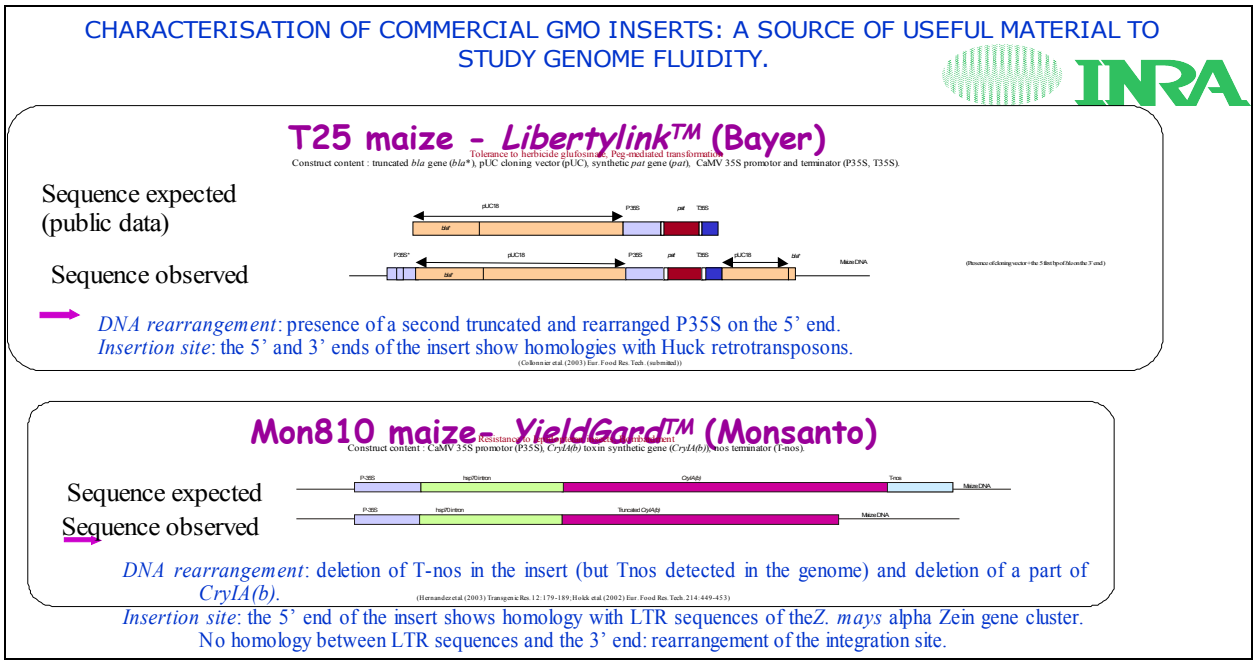

Figure 2: Overview on the difference of the sequence before insertion and the sequence detected in the plant after the insertion by e.g particle bombardment technique. Deletions in the host genome and deletions on the synthetic gene, rearrangements of sequences and novel sequences which do neither belong to the plant genome nor to the synthetic sequences are common features of transgenic plants.

It must be stressed that data on DNA deletions, mutations rearrangements and superfluous DNA of unknown origin is rare as this phenomena are not analyzed during the approval process (i.e.risk assessment of transgenic crops of EFSA). The rare data available is from independent scientists who have analyzed some of the approved sequences several years after approval. A very good overview on this topic is given by WILSON et al. (2004).

A common feature of all these functional aspects in transgenic plants is that new synthetic RNAs are produced from the transgene and in some cases also from unknown sequences around the transgene. These RNAs may be as synthetic as the synthetic transgene itself, i.e. that this kind of RNAs do not occur naturally in any organism on earth. The potential effects of such new RNAs from transgenic crop on the immune system of humans are not investigated and therefore unknown.

2.4 Difference of genes and synthetic transgenes from an evolutionary perspective

Besides functional and structural differences of synthetic transgenes of GM crops and naturally genes of conventional crops, also differences from an evolutionary perspective do exist. From an evolutionary perspective, the creation of genes by enhanced induced mutation (which is rarely used in practice) is "just" speeding up evolution 100 or up to 10,000 times faster than it would occur naturally. It is obvious that such a mutation obtained in the laboratory enhancing the natural mutation rate is likely to occur anywhere on the field around the world on a natural basis by the natural mutation rate. In contrast the creation of synthetic transgenes in plants with combinations of virus promoters, bacterial expression sequences, etc. are not known to occur as a consequence of evolutionary forces. These synthetic genes are not present in any naturally living organism on the earth. They are only present in

genetically modified plants. From the viewpoint of uncertainty, a much higher degree of precaution must be associated with synthetic transgenes.

3 UPTAKE OF FOOD-DNA INTO MAMMALIAN TISSUES

3.1 Introduction

The human health risk of synthetic DNA, RNA in food made from transgenic plants is still neglected. The main argument was that food DNA is fully degraded in the gastro intestinal tract. Cases of uptake from food DNA into the blood of mice as shown by (SCHUBBERT et al. 1994) has been acknowledged but it was seen as rather single event than a general phenomenon (ILSI 2002).

But this viewpoint has changed completely as more and more studies have shown that the uptake of food DNA into the blood and various organs is rather a general phenomenon than the exemption.

A group around Doerfler and Schubbert where among the first ones who showed that orally fed DNA from viruses (M13) reaches the blood stream (SCHUBBERT et al. 1994), peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA (SCHUBBERT et al. 1997). Foreign DNA, orally ingested by pregnant mice, can be discovered in various organs of fetuses and of new-born animals. The M13 DNA fragments have a length of about 830 bp. In various organs of the mouse fetus, clusters of cells containing foreign DNA as revealed by FISH have been identified. The foreign DNA is invariably located in the nuclei (SCHUBBERT et al. 1998). But also subsequent studies have shown similar results (HOHLWEG and DOERFLER 2001, DOERFLER et al. 2001b).

Besides investigations on mice, investigations on live stock animals have brought a more complete picture on this issue.

EINSPANIER et al. (2001) have shown that parts of genes from maize genome can be found in blood and lymphocytes of cows when fed with maize. Similar results for pigs have been found by REUTER (2003). Also in all (exanimated) chicken tissues (muscle, liver, spleen, kidney), parts of maize genome can be detected. Traces of food DNA - could be detected even in the milk (EINSPANIER et al. 2001, PHIPPS et al. 2003) and also in raw pig meat (REUTER 2003). But also in humans, food DNA could be detected (FORSMAN et al. 2003).

The way how food DNA enters the lymph system, blood stream and tissues is not fully understood, but it is thought that "Peyers Patch" play an important role in the uptake of food DNA. Peyer's patches are any of the nodules of lymphatic cells that aggregate to from bundles or patches and occur usually only in the lowest portion (ileum) of the small intestine (www.britannica.com).

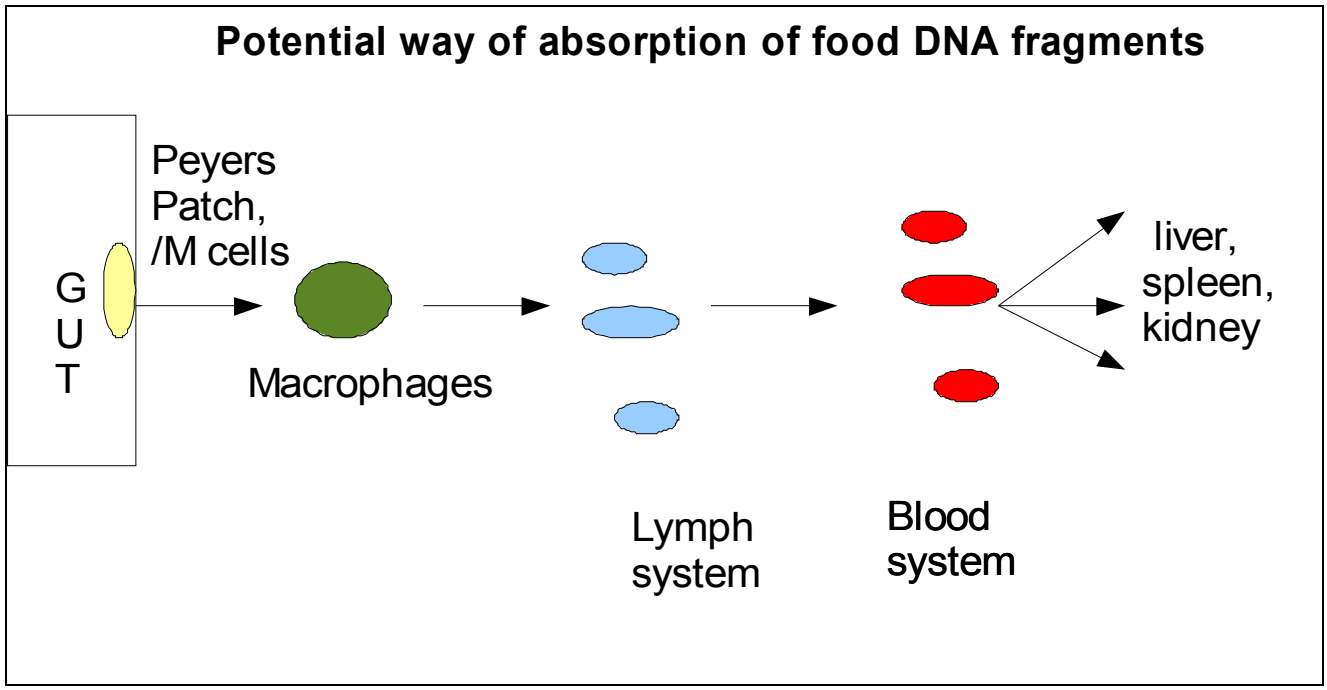

Figure 3: Peyers Patch (i.e nodules of lymphatic cells located in the lowest portion of the small intestine seem to pla a key role in the uptake of food DNA into the lymphatic and blood system

From 2001 it was hypothesized that in contrast to normal food DNA, synthetic food DNA of transgenic plants will be fully degraded, as Einspanier could not detect synthetic DNA but only natural DNA. Research by MAZZA et al. (2005) showed that also fragments of synthetic transgene (from Maize Mon 810) can be detected in the blood and several organs like spleen, liver, kidneys. It is not clear why other groups had not detected synthetic DNA in the body. But it may be du to differences in the sensitivity of the techniques used. But also differences in the primers used may be the cause for the different findings. Maybe some researchers had inadvertently used primers which are across frequent (yet unknown) breaking points of the synthetic gene.

The fact the fragments of food DNA and of synthetic DNA from GM crops are taken up by the blood system is undisputed. But suggestions on the consequences arising from these findings vary widely.

In their conclusions MAZZA et al. (2005) as well as EINSPANIER et al. (2001) denied any risk from the uptake of synthetic sequences in the blood arguing that the uptake of DNA from food is a natural phenomena and that the effect of synthetic food DNA sequences on the body will likely be the same – if any - as effects from conventional food DNA. This is the same viewpoint of ILSI, an industry based knowledge center (ILSI 2002).

But such conclusions have to be marked as assumptions as an investigation on the effect of food DNA has neither been undertaken by MAZZA et al. (2005), EINSPANIER et al. (2001) nor ILSI (2002).

Interestingly researchers from the field of immunology but not from the field of risk assessment of transgenic plants have detected sequence specific effects of external DNA independently of the way it was delivered (intragastric, injected or fed, see chapter 4). RACHMILEWITZ et al. (2004) looked at the immunostimmulatory effect of DNA from probiotic bacteria and looked also at the presence of the DNA in the blood and organs of mice. He concluded that the localization of this bacterial DNA in these organs coincided with its immunostimulatory activities.

Thus it seems to be more likely that the presence of other food DNA and synthetic food DNA detected in various organs and the blood will also coincide with yet uninvestigated and therefore unknown immunomodulatory activities.

3.2 Presence of Circulating RNA

As described above (Chapter 2) transgenic plants do also produce synthetic RNAs (Roundup-ready soybean (RANG et al. 2005, maize NK603 (EFSA 2003)). Impacts from these RNAs on the immune system can not be ruled out, as RNAs play a very important role in the regulatory network of mammals (KENZELMANN et al. 2006). But the presence of synthetic RNAs in the blood of or organs unlike the food DNA has not been investigated so far.

Many researchers questioned the possibility of stable RNAs because of the presence of ribonuclease which degrades rapidly RNAs in the blood and

because of the known low stability of RNAs. But as the following overview shows RNA is surprisingly stable in the blood.

> *The existence of circulating RNA is a remarkable finding because RNA is more labile than DNA and ribonuclease is known to be present in blood . At present, the exact mechanisms that protect circulating RNA are still unknown. The RNA may possibly be complexed to lipids, proteins, lipoproteins, or phospholipids bound with DNA in nucleosomes (9, 12); or protected within apoptotic bodies or other vesicular structures (Tsui et al. 2002)*

Furthermore, cell-free circulating fetal nucleic acid RNA can be detected in the bloodstream of the mother. But few minutes after birth, the RNA could not be detected in the bloodstream of the mother. The fact that cell-free RNA is detected leads to the conclusion that the RNA itself is acting as communication unit. This is supported by the fact that some of the detected fetal RNA in the bloodstream of the mother are so called non-coding RNAs, where no protein could be detected (Ng et al. 2003).
The authors conclude that:

> *"Finally, because hPL and β-hCG are both hormones, our data may have broader implications in the field of* ***endocrinology****. If our results can be generalized to other hormone systems, then a radically new approach for studying and assessing endocrinological disorders may be possible (Ng et al. 2003).*

Moreover, not only cell-free RNA but also cell-free fetal DNA is found in maternal plasma (SEKIZAWA et al. 2003, SEKIZAWA et al. 2004, FARINA et al. 2004a, FARINA et al. 2004b, MASUZAKI et al. 2004) - the function of cell-free DNA is not clear.

4 FOREIGN AND SELF DNA/RNA AND THE IMMUNE SYSTEM

4.1 Introduction – Innate Immune system and Recognition of universal Patterns

The immune system is divided into innate and adaptive immunity. In new born babies the innate immune system provides the first line of host defense against invading microorganisms before the development of adaptive immune responses. Innate immune responses are initiated by germline-encoded pattern recognition receptors (PRRs), which recognize conserved molecular patterns (pathogen-associated molecular patterns, PAMPs) of microorganisms. These so called pattern-recognition receptors (PRRs) are quite different from the large repertoire of rearranged receptors in adaptive immunity . PAMP are though to be highly conserved structures among many pathogens. Structures which are needed by the pathogen to survive and which cannot be easily replaced by other structures. Different PRRs react with specific PAMPS, show distinct expression patterns and activate different signaling pathways. PAMPs are good targets for the innate immune system to discriminate between self and nonself with limited numbers of PRRs (AKIRA et al. 2006).

Proteins and interestingly nucleic acid can act as pathogen-associated molecular patterns. Why nucleic acid is identified by human (mammalian) pattern-recognition receptors (PRRs) is still not fully clear, but some argue that microbial nucleic acids represent a uniform molecular pattern, allowing recognition independently of continuous evolutional changes to the outer membrane or capsid components (PAWAR et al. 2006).

Specific nucleic acid sequences seem to be evolutionary conserved and represent a universal code which is identified as sequence from a pathogen by the innate immune system. This universal knowledge is passed from generation to generation.

The current research on foreign (= not self) nucleic acid and the immune system focuses on the effect from nucleic acid from micro organisms. To date it is clear that the immune system recognizes DNA/RNA of viruses, microorganisms and self DNA or RNA. The delivery of DNA can be by food or various forms of infections. Even synthetic DNA/RNA can trigger immuno

modulatory actions. However we could not find any research report which has addressed the question if the immune system of mammals or humans is able to interact with DNA/RNA from plants. At least for C. elegans it was shown that small interference RNA (siRNA) from plants can silence genes in C. elegans by feeding(KAMATH and AHRINGER 2003).

But we could find one research report which has identified a shrimps RNA as a food allergen for humans. So from this picture it is highly likely that the interaction with foreign (=non self) DNA is a general feature of the human immune system.

In the following we very briefly review the current state of knowledge on human receptors recognizing nucleic acid from food, pathogens or self.

Two different ways how the immune system detects DNA/RNA from viruses, microorganisms and self DNA have been identified so far:

1. Recognition in the endosome by Toll-like-receptors TLR
2. Recognition in the cytoplasma by retinoic acid-inducible protein1 (RIG-1) and MDA5

Figure 4 gives an overview on already identified receptors for foreign DNA, RNA or other nucleic acid.

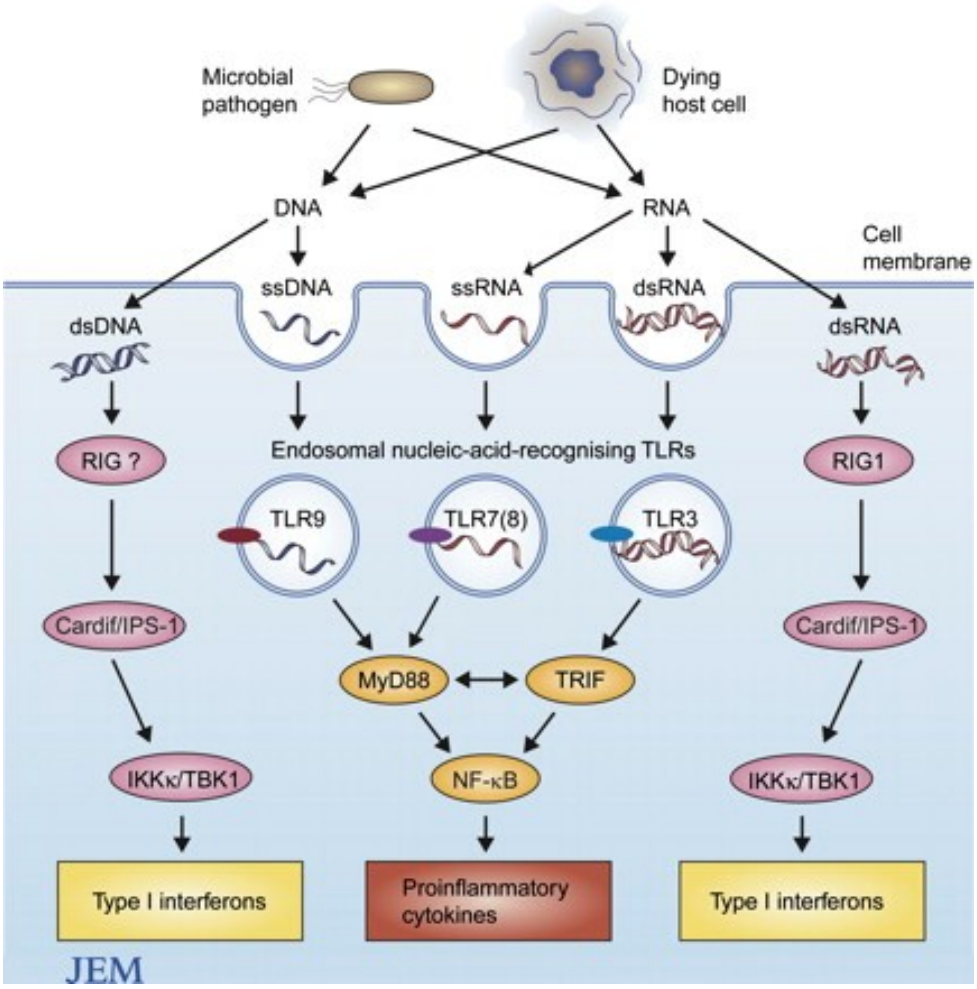

Figure 4: Nucleic acid recognition pathways in innate immune cells. (WAGNER and BAUER 2006 for details see Textbox below)

Nucleic acid recognition pathways in innate immune cells. Both pathogen-derived RNA or DNA and host-derived mammalian RNA or DNA are sensed via TLR and TLR-independent recognition pathways. Upon endosomal translocation, viral dsRNA, microbial or mammalian ssRNA and ssDNA are recognized by endosomally expressed TLR3, TLR7, (8) and TLR9, respectively. After ligation of TLR7, TLR8, and TLR9, the adaptor molecule MyD88 is recruited and drives the production of proinflammatory cytokine genes or type 1 interferon genes. TLR3 ligation triggers type 1 interferon genes via the adaptor protein TRIF. Viral dsRNA is also sensed by RIG-1 (retinoic acid inducible gene 1), which was recently shown to recruit Cardif/IPS-1, a new CARD-containing adaptor protein. Cardiff /IPS-1 in turn interacts with Ikk

alpha/ß/gamma kinases and thus activates IRF3. Mammalian DNA triggers type 1 interferon production by an ill-defined signal pathway. Whether the dsDNA recognition receptor belongs to the RIG family is not yet known. (source: http://www.jem.org/content/vol203/issue2/images/large/481fig1.jpeg) (WAGNER and BAUER 2006)

4.2 Recognition in the Endosome by Toll-like-receptors (TLR)

4.2.1 Overview on TLRs

One of the mayor pathways to detect foreign or self DNA/RNA in mammals is via Toll-like-receptors (TLR). TLR are evolutionary conserved from the worm C. elegans to mammals.

"Toll, the founding member of the TLR family, was initially identified as a gene product essential for the development of embryonic dorsoventral polarity in Drosophila. Later, it was also shown to play a critical role in the antifungal response of flies" (AKIRA et al. 2006). (This is a very good example of multi functional properties of genes and proteins and how long it takes that very different properties of the exact same gene or gene product are identified).

In the search for human homologs to Drosophila's Toll, a new family of pattern-recognition receptors was discovered that can recognize a great variety of pathogens, including viruses, fungi, and bacteria. The members of the Toll-like receptor (TLR) family recognize conserved molecular patterns, including peptidoglycans, lipopolysaccharides (LPS), and, most interestingly, nucleic acids" (PAWAR et al. 2006).

To date, 12 members of the TLR family have been identified in mammals (AKIRA et al. 2006). The other authors suggest different 11 TLRs (PAWAR et al. 2006).

Table 1 gives a short overview on TLRs and their ligands.

Table 1: Pattern recognition of TLRs

TLRs	ligands
TLR1	Triacyl lipopeptides (in association with TLR2) (bacteria)

	peptidoglycan, lipoprotein, lipopeptides, atypical LPS(bacteria)
TLR2	zymosan, phospholipomannan (fungi) GPI anchor (protozoa) hemagglutinin protein (virus)
TLR3	Poly(I:C), **dsRNA** (virus) LPS (bacteria), endogenous **mRNA**
TLR4	mannan, glucuronoxylomannan (fungi), Glycoinositolphospholipids (protozoa) Envelope protein (virus)
TLR5	Flagellin (bacteria)
TLR6	diacyl lipopeptides (in association with TLR2) (bacteria)
TLR7/TLR8	Synthetic imidazoquinoline-like molecules, **ssRNA** (virus)
TLR9	**CpG DNA** (bacteria, protozoa, virus), hemozoin (protozoa) profilin like molecule (protozoa)

TLRs recognize pathogens either on the cell surface or in the lysosome/endosome compartment. Interestingly those receptors that recognize nucleic acids are not expressed on the cell surface unlike other receptors of the TLR family as TLR2, TLR4, TLR5, TLR11which recognize lipopetides, lipopolysaccaride, flagellin and proppelin and are expressed on the cell membrane (PAWAR et al. 2006, WAGNER and BAUER 2006). Nucleic acid recognizing TLRs are expressed in the intracellular lysosome/endosome compartment of cells. After phagocytes internalize viruses or virus-infected apoptotic cells, viral nucleic acids are released in phagolysosomes and are recognized by these TLRs. When TLRs bind to nucleic acid they trigger different signaling pathways. Many of these pathways consists of the toll-interleukin 1(IL-1) receptor (TIR) domain. Most of these pathways results in the expression of type I interferon, cytokines but also chemokines. As the signalling pathway is very complex, more details on the signaling shall be obtained from reviews of the TLR-family (PAWAR et al. 2006, AKIRA et al. 2006, WAGNER and BAUER 2006). Here we only want to give an overview which types of nucleic acids are recognized by the different TLRs.

4.2.2 TLR3

TLR3 recognizes double stranded RNA (dsRNA) from e.g viruses. TLR3 is also involved in the recognition of polyinosine-deoxycytidylic acid (poly I:C), a synthetic analog of dsRNA which is generated during viral replication (UEMATSU and AKIRA 2006). In vitro experiments show that TLR3 is also a

receptor for endogenous mRNA and small-interference RNA (PAWAR et al. 2006). TLR3 is expressed on myeloid dendric cells of the immune system. TLR3 is the only nucleic acid-specific TLR expressed by nonimmune cells, e.g., glomerular mesangial cells in mice and humans (PAWAR et al. 2006) and human vascular endothelial cells like human indestinal microvascular endothelial cells (HIMEC), human umbilical vein endothelial cells (HUVEC) (HEIDEMANN et al. 2006).

4.2.3 TLR7/TLR8

TLR7 and TLR8 are close relatives. TLR7 bind to GU rich single stranded RNA (ssRNA) of viruses. TLR7 is expressed on plasmacytoid and myeloid dendric cells as well as B cells. TLR8 is only found on myeolid dendric cells and macrophages. TLR7 and TLR8 are apparently absent from nonimmune cell types (PAWAR et al. 2006). But in his review HEIDEMANN et al. (2006) reports that Gunzer et al. has detected expression of TLR7 in murine endothelian cells in vivo and in vitro. Host (self) RNA can also be detected by TLR7 receptors as WAGNER and BAUER (2006) point out.

4.2.4 TLR9

KRIEG (1996) was among the first ones who detected the activation of immune cells by CpG DNA of procaryotic organisms. Since then there has been a lot of research on TLR9. Therefore TLR9 is one of the best studied TLR which interacts with nucleic acids. The following review gives only a short overview of some main findings.

CpG-DNA is defined as DNA oligodeoxynucleotide (ODN) sequences that include a cytosine–guanosine sequence and certain flanking nucleotides, which have been found to induce innate immune responses through interaction with TLR9 (www.nature.com/nri/journal/v5/n6/glossary/nri1630_glossary.html). These CpG-Islands are G/C-rich DNA-regions, which indicate the presence of a close gene. Informative DNA-regions contain significantly more G- and C-nucleotides compared with non-coding DNA-regions (THEODOR DINGERMANN and ILSE ZÜNDORF 1999). "CpG" stands for cytosine and guanine separated by a phosphate which links the two nucleotides together in DNA. (en.wikipedia.org/wiki/CpG_site, 04.11.05)

CpG dinucleotides are underrepresented and selectively methylated in vertebrate DNA, but are present at the expected frequency and are unmethylated in bacterial DNA (Cardon et al. 1994 in RAY and KRIEG 2003) Frequency of occurrence in bacterial DNA is of ~ 1 in 16 dinucleotides, and less than 5 % of the cytosines in these dinucleotides are methylated, whereas in vertebrate genomes they are occurring at a frequency of ~ 1 in 125 dinucleotides and 70-90 % of the cytosines in these dinucleotides are methylated, which greatly diminishes their immunostimulatory effects (7, 10 in SILVERMAN and DRAZEN 2003) CpG motifs of bacterial DNA are known to be potent activators of innate immunity (OBERMEIER et al. 2005).

TLR9 recognize unmethylated cytosine-guanosine dinucleotide (CpG) motifs in ssDNA or dsDNA. Such CpG DNA motifs do occur more frequently in bacteria and viruses. But also mammal DNA consists of CpG DNA motifs. TLR9 is expressed on B cells and plasmacytoid dendric cells in humans. In mice TLR9 is also expressed in monocyte/ macrophages and myeolid dendric cells (PAWAR et al. 2006). HEIDEMANN et al. (2006) report that TLR9 is also expressed in endothelilal cells and human colonic cells have been shown to respond to bacterial CpG DNA stimulation. But also pulmonary endothelial cells of mice and rats do express TLR9.

4.3 TLR Independent Recognition of DNA/RNA

4.3.1 Recognition in the Cytoplasma by Retinoic Acid-Inducible Protein1 (RIG-1)

Toll-like independent receptors which do recognize nucleic acid in the cytoplasma have been identified recently. The retinoic acid-inducible protein1 (RIG-1) seems to be responsible for TLR independent response when cells are challenged with viral DNA or RNA (WAGNER and BAUER).

The helicase RIG-I binds dsRNA and is involved in the type I IFN response to virus infection (LOPEZ et al. 2006). HEIDEMANN et al. (2006) report that NOD2/CARD15 might be involved in the TLR independent recognition of nucleic acids. Further studies have to be performed to fully understand the TLR independent recognition of DNA or RNA. So more TLR independent receptors might be up on the horizon.

4.4 Impacts of Foreign (= non self) DNA on the Immune System

In chapters 4.2,and 4.3 the receptors to recognize DNA or RNA from pathogens or host has been briefly described. In the following we want to give a very short overview of consequences of DNA or RNA on the immune system.

4.4.1 Pathogenic effects

For long it was believed that bacteria or their proteins (endotoxins) are the main cause for triggering diseases or infections. But more and more evidence has been aggregated which shows that also DNA or RNA of the pathogens alone is able to trigger the disease. Here we give only a random selection of what is known on pathogenic effects of foreign (=non self) DNA/RNA.

Quite early a team around Arthur Krieg showed that CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. (SCHWARTZ et al. 1997).

In 1997 PISETSKY concludes that immunologic activities of bacterial DNA resemble those immunologic activities of endotoxin and adds

> *" The categorization of DNA as an immune activator contrasts with previous portrayals of DNA as immunologically uniform and inert"*

He also suggested that, in general, foreign nucleic acids can stimulate immune responses because of structural microheterogeneity.

In a commentary SUCH et al. (2005) point out that

> *"DNA is not only representative in itself of the presence of bacteria (either viable or non-viable) in our patients, but induces similar immunological changes as endotoxin or viable bacteria".*

In their study SUCH et al. (2002) describe the presence of bacterial DNA in the blood of patients with cirrhosis and determined its role in triggering immune response in a follow up research (FRANCES et al. 2004).

Another team showed that bacterial DNA containing unmethylated CpG motifs induces meningitis, and indicates that this condition is mediated in vivo by activated macrophages (DENG et al. 2001).

PAWAR et al. (2006) describe how several kidney diseases like Lupus nephritis, Glomerulo nephritis Renal vasculitis are linked to the direct involvement of DNA or RNA. They point out that "circulating viral RNA usually complexed in immune complexes that provide resistance against rapid RNAse digestion" is thought to be involved in kidney diseases like Glomerulo nephritis.

4.4.2 Allergenic Effects

Surprisingly almost 20 years ago researchers identified a food RNA of shrimp to be a mayor allergen for humans (NAGPAL et al. 1987). Which shows that not only food-DNA from micro organisms is interacting with the immune system of humans. There is also evidence that immunostimulatory sequences may temporary attenuate allergenic diseases like asthma (SILVERMAN and DRAZEN 2003).

4.4.3 Beneficial Effects

Most of the research on foreign (= not self) nucleic acid and the immune system focuses on detection of nuclei acids of pathogens by the immune system. But there is also research showing that food DNA of beneficial bacteria (e.g lacto bacillus) have a beneficial effect.
RACHMILEWITZ et al. (2004) investigated the protective effects of probiotics. They concluded that:

> *"The protective effects of probiotics are mediated by their own DNA rather than by their metabolites or ability to colonize the colon. TLR9 signaling is essential in mediating the anti-inflammatory effect of probiotics, and live microorganisms are not required to attenuate experimental colitis because nonviable probiotics are equally effective."*

Later studies confirmed the initial findings from 2004 (LEE et al. 2006)
RACHMILEWITZ et al. 2004 ,also investigated the uptake of probiotic DNA and found a higher rate of uptake of DNA by intragastric delivery compared to intrarectal delivery. A very important finding of this study is that the

localization of this bacterial DNA in these organs coincided with its immunostimulatory activities, Pprobiotic DNA was detected in the liver and spleen after daily i.g.administration of irradiated probiotics, which was initiated 10 days prior to induction of colitis with DSS, and for 7 days thereafter. The authors conclude:

> *„Taken together, these data indicate that most of the probiotic DNA is absorbed from the upper gastrointestinal tract and most probably acts systemically as occurs with sc injection of other types of immunostimulatory DNA (e.g., ISS-ODN).*"

Besides effects of probiotic DNA from probiotic bacteria many other beneficial immuno modulatory effects are investigated. Synthetic ODNs that bind to TLR9 are proposed for several treatments e.g. some cancer diseases (WANG et al. 2006).

4.5 Sequence specifity

4.5.1 Introduction

The sequences in question which interact with the immune system are oligodeoxinucleotides(ODN). In several investigations several sequences have been tested on their immunostimulatory (ISS-ODN) or inhibitory ODN effect. After the first detection of immunogenic effects of CpG DNA by e.g. SCHWARTZ et al. 1997) many questions remained like:

- Which sequence of the DNA is immunstimulatory (beneficial), which is immunogenic (pathogenic) or neutral?
- How does the mammalian immune system distinguish between self and foreign DNA.
- Do sequences have the same immuno modulatory effect across species or is there also a species specific activation of the immune system.

4.5.2 Differences between inhibitory and stimulatory sequences

It seems that there are only narrow differences when a given DNA/RNA acts in a neutral, beneficial or pathogenic way to the immune system. Only certain classes of ISS-ODNs are able to contribute to the beneficial effect in tissues with ulcerative colitis (RACHMILEWITZ et al. 2006).

Also synthetic ODNs are able to reduce the stimulatory effect of CPG DNA. Surprisingly the difference between stimulatory and inhibitory sequences can differ by as few as two bases as research by ASHMAN et al. (2005) shows. The identified optimal synthetic inhibitory sequence is 11 bases short. The sequences is described as follows: "5´ CC/x/notC/notC/x/x/GGG/x/ or CC/x/notC/notC/x/GGG/x/x/ where x is any base". The authors conclude that three areas are critical. Between these areas are "space sequences" where not a specific base but a specific number of bases is important for the sequence to act inhibitory.

4.5.3 Distinction between Foreign and Self DNA by the immune system

One of the most interesting questions is why the immune system is only activated by bacterial DNA and not by the host own DNA.. Many researchers working in this field laid their main interest on interactions of CpG DNA and mammalian TLR9 receptor. The reason why bacterial CpG motifs but not vertrebrate CpG motifs is binding to TLR9 is still not fully understood. For long time it was believed that methylation of CpG motifs inhibits binding of self CpG motifs. But STACEY et al (2003) have identified a mixture of CpG methylation, general CpG surpression, inhibitory motifs such as GGAGGGG which altogether seems to modulate the binding of TLR9 to CpG DNA. They conclude that

> *"The immunostimulatory activity of DNA is determined by the frequency of unmethylated stimulatory sequences within an individual DNA strand and the ratio of stimulatory to inhibitory sequences (STACEY et al. 2003).*

Another explanation why unmethylated CpG motifs which are present in mammalian genomes do not induce a response like bacterial CpG motifs is given by GURSEL et al. (2003). The end of chromosomes in eucariotic chromosomes are called **telomers** which consist of large numbers of repeats of the sequence TTAGGG. Telomers are involved in a high number of key regulations in the cells, like cell cycle regulation, cellular aging, transcriptional regulation etc. Telomeric G-rich repeats are present at high frequency in eukaryotes but rare in bacterias. The TTAGGG down regulate the response to CpG DNA. GURSEL et al. (2003) argue also that the immunosupressive effect of TTAGGG was highly specific and did not effect mitogen induced cytokine or Ig production.

"Finally pure DNA from telomers was more surpressive than DNA from nontelomeric regions of the genome"GURSEL et al. 2003.

GURSEL et al. (2003) observations show also that the inhibitory sequences of the telomers act in low concentrations. They believe that sequences which are able to from G-tetrads like the TTAGGG sequence of telomers are able to suppress the immunostimulatory effect of CpGs.

4.5.4 Species specific activity

The particular DNA sequences that provoke immune response vary between species (AKIRA et al. 2006). The effects of ISS-ODNs on the immune system depend on the specific sequence, animal species, dose, time course, and route of delivery. For example, the sequence GACGTT activates innate immune cells of the mouse much more efficiently than similar cells from humans. The sequence, GTCGTT confers optimal immunostimulatory effects in human cells (KRIEG et al. 1995).

4.6 Transient activity

Many effects of foreign (=non self DNA) seem to be transient as other effects of food ingredients like proteins and secondary plant metabolites. The attenuation of asthma by immuno stimulatory sequences is transient (SILVERMAN and DRAZEN 2003) as well as CpGs act only a few weeks against viral patterns (KLINMAN 2004). This fits very well in the picture which is drawn by Doerfler in a personal review of his 30 years work on DNA, DNA mehtylation and uptake of foreign DNA (DOERFLER 2005). Foreign nucleic acid which is taken up by the gastro intestinal tract, is not integrated in the host genome, but diminishes after a few days to weeks, after exposure (DOERFLER 2005).

Some researchers (ILSI 2002) have tried to limit the discussion about risks of food DNA detected in blood to the issue of horizontal gene transfer. In their opinion there is only a risk if the whole gene of the food is inserted into the human genome and expresses the novel protein. They further argue that this has never been the case, and they therefore conclude that there is no risk associated with synthetic food DNA from transgenic crops. But this assumption is a too narrow interpretation of potential interactions between

food DNA and the immune system which rules out other forms of interactions than integration by horizontal gene transfer.

As foreign DNA has a transient role on the immune system the role of food DNA is not to be integrated into the genome but to transiently kick on some regulatory processes. As with other ingredients of food like proteins, carbohydrates secondary plant metabolites also DNA seems to fulfill a transient task, which diminishes after some days to weeks.

5 DISCUSSION:THE ROLE OF FOOD DNA/RNA IN THE RISK ASSESSMENT OF TRANSGENIC PLANTS

5.1 Current state of risk assessment

The current risk assessment on long term consequences on human health is based on

- Comparative assessment of mayor food ingredients
- Short term toxicological studies with the Protein
- Sometimes 90 day whole feed toxicological studies

There are many short comings in the current risk assessment of genetically modified plants which have been described in more detail by others (MILLSTONE et al. 1999, PUSZTAI et al. 2003, MÜLLER 2002, MÜLLER 2004, MUELLER et al. 1999, SPÖK et al. 2002, MILLSTONE 2002, SPÖK et al. 2003).

Unfortunately the European Food safety authority responsible for the risk assessment of genetically modified plants, has resisted to acknowledge these short comings, and the EC Commission has based all its approvals on the risk assessment of the EFSA so far.

The documents in which the risk assessment process is documented are called "opinions".

So far EFSA has only provided positive opinions on genetically modified plants concluding that the genetically modified plant is as safe as a conventional crop for human consumption and environmental release.

All these opinions are based on risk assessment data for short term or in some cases subchronic toxicity studies. No opinion is based on data from long term toxicity experiments or so called chronic toxicity studies i.e 24 months studies which are obligatory in the approval process of pesticides. Although the legal requirement for long term assessment of human health effects is explicitly described in Directive 2001/18/EC (see Annex II of Directive 2001/18/EC) all approvals by the EC commission were made lacking long term toxicity studies.

As described above the main focus of the risk assessment of genetically modified plants by EFSA is on comparison of chemicals compounds and on proteins.

5.2 Exceptional Case: Touching the Risks of synthtetic RNAs in the assessment of NK603 by EFSA

5.2.1 Introduction

So far risks of chronic toxicity and risks by the synthetic DNA or RNA of the genetically modified plants have not been addressed by EFSA in their risk assessment. But there is one exception of the rule. This is the risk assessment of the Roundup resistant genetically modified maize NK603. It was the first risk assessment of a genetically modified plant undertaken by the newly established European Food Safety Authority (EFSA) in 2003. In this risk assessment EFSA slightly touched the issues of risks associated by RNAs of sequences of unknown origin, which are present in the genetically modified maize NK603. In all other subsequent risk assessments of genetically modified plants EFSA returned to the narrow focus on proteins and chemical compounds, and did not touch the issue of synthetic RNAs.

5.2.2 Genetically modified maize NK 603: EFSA touches slightly Risks from RNA

Genome scrambling (insertion site mutations and genome wide mutations) is a quite common phenomenon or by product of transgenic plants (WILSON et al. 2004). Transgene insertion do not only result in the insertion of synthetic transgenes but also in deletion and/or insertion of extra fragments of unknown origin (see Chapter 2).

This is also the case in the genetically modified maize NK603. This transgenic maize contains extra fragments which do neither belong to the plant DNA nor to the DNA of the synthetic gene (insert) which has been shot into the maize genome by particle bombardment. As the stop sequence of the synthetic transgene in the maize NK603 is not working properly the translation process also produces new RNA by so called read through.

In the assessment of NK 603 EFSA stated that these RNAs from the synthetic gene produced in NK603 does not give rise to any concern because:

"… the RNA fragment observed in the product of the RT PCR amplification is not expected to have a regulatory function as described for micro RNAs which are short RNAs between 21 and 23 bp long derived from the processing of longer RNAs of around 70 bp (Jones, 2002). This is much shorter than the RNA fragments amplified from NK603."
(EFSA 2003)

In other word the extra fragment is too long to have any regulatory function, but shorter fragments may pose a risk or give rise for a concern.

The EFSA argumentation in 2003 that only short RNAs between 21 and 23 bp do have a regulatory function is wrong. Even in 2003 several RNA databases showed that also long fragments of RNA show regulatory function.

KENZELMANN et al. (2006) describe the current situation as following: Non coding RNAs range from 21-25 (SI RNA and miRNA) to 100 –200 nucleotides for small RNAs up to 10.000 nucleotides for RNAs involved in gene silencing. So any RNA regardless of its length is able to have regulatory function.

Thus the reason to deny any risk from the synthetic RNAs in the genetically modified maize NK603 is not scientifically justified.

5.2.3 Genetically modified oil seed rape GT73: EFSA does not Touch Risks from RNA, to reach a positive opinion on the safety of GT 73

There are remarkable differences in assessing extra genome sequences between the assessment of NK 603 by EFSA and GT 73 by EFSA

In all other opinions EFSA is not referring to any risks associated by synthetic RNA or DNA fragments detected in the transgenic plants.

Even in subsequent risk assessment of GT73, EFSA do not again evaluate the risk associated with fragments of unknown origin in the transgenic plant.

When describing the synthetic transgene in GT73, (Section 2.2.2) EFSA states

"The sequencing of 3' and 5' flanking regions revealed that 40 base pairs (bp) of parental (Westar) DNA is

absent from GT73, and that GT73 contains 22 bp of DNA adjacent to the 5' insert/plant junction which is not present in Westar".
(EFSA 2004)

As in many cases it is sometimes more important not to listen too much what has been said but to focus on that what has NOT been said.

(EFSA).

This citation box is empty to draw the attention to that what EFSA did not say/analyze in the risk assessment of GT 73

EFSA did not mention that the extra fragment present in GT 73 of a length of 22 base pairs fits precisely in the definition of "regulatory micro RNA" 21-23 (bp) – which could pose a risk as acknowledged by EFSA in the risk assessment of NK603 (see citation in Chapter 5.2.2).

But also while assessing the stacked event genetically modified maize 1507xNK603 in 2006 i.e only three years later EFSA does not mention its initial findings on RNAs and solely focus on proteins, as following citation shows.

„The insert in NK603 does include some molecular rearrangements at one end of the insert and also includes a fragment of chloroplast DNA. These rearrangements and the insertion of chloroplast DNA do not lead to new traits and are not considered to pose a safety risk. In the unlikely event that a new peptide or protein is produced as a consequence of the insertion

event, bioinformatics analysis showed that these would have no homology to known toxins or allergens."(EFSA 2006)

EFSA hide its own findings from 2003 on new RNAs detected in the genetically modified maize NK603 in its initial assessment, and assumes that there is no protein made from rearrangements. The word "trait" in the sense EFSA is using means protein. EFSA does not touch potential risks of new traits by a regulatory RNA, maybe knowing that its initial exclusion of risks from RNA longer than 22 base pairs is not scientifically sustainable, as shown above. So far every risk assessment of genetically modified plants undertaken by EFSA resulted in a positive opinion in favor of market approval, i.e in favor of the applicant. In 2006 EFSA would get in mayor trouble stating the RNAs longer than 22base pairs are without regulatory functions. EFSA circumvented this problem by hiding its initial statement and could thus reach a positive opinion in favor of market approval, i.e in favor of the applicant but in disadvantage for the customers who may ingest this maize 7 days a week for their whole lifespan.

5.3 A short historical review on the risk Assessment of GM in comparison with Findings on immunogenic effects of Foreign DNA

Quite early in 1987 the relevance of food nucleic acid to the immune system has been identified. NAGPAL et al. (1987) detected that mRNA of shrimps is a mayor food allergen. In 1995 the genetically modified roundup resistant soybean was approved in Europe, without taking notice of risks associated with nucleic acids. In the same year KRIEG et al. (1995) KRIEG published his findings on the activation of immune cells (receptor TLR9) by CpG DNA and PISETSKY (1997) stated that in its immunologic activities, bacterial DNA resembles endotoxin. In the same year DNA has been detected to trigger inflammation (SCHWARTZ et al. 1997).

All this evidence appeared 6 years before EFSA started with the risk assessment on the genetically modified maize NK603.

Since 1997 the body of evidence has been increased significantly as this review shows. In 2003 when EFSA started its risk assessment it was already established that foreign DNA can trigger several diseases (e.g. SUCH et al.

2002a, DENG et al. 2001,) and that there are several receptors beside TLR9 in the human immune systems that recognize foreign DNA (ALEXOPOULOU et al. 2001, HEMMI et al. 2002).

EFSA was and is still very silent on this issue as the recent risk assessment opinion of EFSA from Juli 2006 shows (EFSA 2006). Up to now EFSA ignores effects from synthetic sequences of DNA from genetically modified plants.

5.4 The Importance to Incorporate Risks of Synthetic DNAs and RNAs into the Risk Assessment of Genetically Modified Plants

To summarize the global picture on synthetic DNA and the Immune system

- Synthetic Genes (sequences) of transgenic plants are different to genes (sequences) from conventional plant breeding.
- The synthetic genes of genetically modified plants are unique, man made constructs and do not occur in any living species on the earth.
- These sequences are apparently new to the immune system and the ecosystem
- DNA fragments of genes from food are remarkably stable (more stable than very stable proteins) and pass the gastro intestinal tract and can be found in various tissues, like blood, lymphozytes, liver, spleen, kidneys.
- Several sequences of RNA , DNA, CpG DNA are recognized by the human immune system. The effect of the DNA/RNA is sequence specific.
- The properties of foreign DNA reaches from immunostimulatory, to inhibitory, to immunogenic (pathogenic) activity.
- In vivo experiments have shown, that the activity of foreign (= not self) DNA is correlated wit the presence of the DNA in various tissues like liver and spleen, after intragastric delivery.
- Thus food DNA found in various tissues may very likely also exhibit a yet unknown activity.
- Interactions **of foreign DNA are transient** like other interactions of food (foreign) proteins, secondary plant metabolite. A gene transfer of foreign (food) DNA into the host genome (humans) is not very likely.

- Chronic exposure of synthetic sequences from transgenic plants which are new to the immune system, may lead to yet not investigated and therefore unknown health impacts.
- The risk assessment of genetically modified plants has to address risks from synthetic RNAs and DNAs on the immune system to avoid that mayor aspects of potential harm has been ignored and pose a harm to human health

This is in line with he review of Doerflers on laboratory findings over the past 30 years. He states:

> *"Taken together, the results of this series of investigations indicate that foreign macromolecules, particularly the very stable DNA, can survive in the gastrointestinal tract at least transiently in small amounts and in fragmented form and can get access to various organ systems of the mouse. Even stable proteins survive only for a very short time in the gastrointestinal tube. We have not found any evidence for the entry of foreign DNA into the germ line, nor could we demonstrate transcription of foreign DNA in any of the organ systems tested. It is not known whether a tiny proportion of the thus persisting DNA may find entry into the genome of a rare defense cell and remain there with unknown functional consequences. These questions will be worth pursuing (DOERFLER 2005)".*

These findings indicate that RNA/DNA sequences of food derived from plants or animals may also interact with the immune system as it was extensively demonstrated for DNA/RNA sequences from micro-organisms and viruses.

All genetically modified plants so far assessed by EFSA do contain one or more sequences derived from micro-organisms or viruses. These sequences had been further manipulated to increase expression in transgenic plants. As

it was shown that those sequences from micro-organisms can cause inflammation in various organs, the effects of synthetic DNA/RNA of genetically modified plants on the human immune system must be part of any risk assessment of genetically modified plants.

It seems that the sequences of unknown origin and synthetic sequences may interfere with ancient universal patterns which are recognized by the immune system. As RACHMILEWITZ et al. (2004) reveal the localization of the intragastric deliverd bacterial DNA in organs of mice coincided with its immunostimulatory activities. This finding clearly indicates that synthetic fragments detected in mammalian blood and tissues (MAZZA et al. 2005) will also correlate with its -yet unknown - immunostimulatory activities.

Food DNA is able to pass the GIT and can be detected in several tissues and cells like the blood, lymphocytes, liver, spleen, kidneys.

6 OUTLOOK

In a review KENZELMANN et al. (2006) state that there are more conserved ncRNA regions on the genome than protein coding DNA sequences, which highlight the importance of nucleic acid in the regulatory network of humans. Recent research shows that RNA plays a key role in building complex regulatory networks (MATTICK 2005, KENZELMANN et al. 2006). The interaction of non-coding DNA (RNA genes, introns from protein coding genes, intron from RNA genes) with the cell is hardly understood. The main focus in basic research has long been on proteins. The role of RNA has long been underestimated in science. Now the focus has got a dramatic shift from proteins to RNAs and its abundant regulatory functions. The European Food Safety Agency (EFSA) has so far resisted to take notice of these dramatic changes in cell biology and to incorporate these new findings into the risk assessment of genetically modified plants. The focus in the risk assessment of transgenic plants is still on proteins. Potential effects on the regulatory network of humans by the synthetic DNA and RNA of genetically modified plants are ignored for unknown reasons. We hope that this report may help to focus more on potential effects of synthetic DNA and RNAs of genetically modified plants on the human immune system.

"The failure to recognize the importance of introns may well go down as one of the biggest mistakes in the history of molecular biology"
John S. MATTICK Director of Institute of Molecular Bioscience, University Queensland (Australia) (see Gibbs 2003)

While risk assessment and basic understanding of molecular biology are closely linked we predict that:

"The failure to recognize the importance of RNA produced by non-coding regions of DNA (introns, RNA genes, pseudogenes etc.) may well go down as one of the biggest mistakes in the history of risk assessment of transgenic plants. The human genome exhibited the greatest number of non-coding RNA sequences. The consequence is that humans are possibly the most sensitive species to novel synthetic RNAs and DNA produced by genetically modified plants".

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