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Safety issues associated with the DNA in animal feed derived from genetically modified crops. A review of scientific and regulatory procedures

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Background

The world's population is expected to increase by a third in the next 30 years, whilst land for crop and livestock production will continue to decline with further urbanisation. It is thus inevitable that intensive agriculture will continue in order to ensure adequate food supplies to provide a satisfactory diet for an ever-increasing number of people. This is contrary to the views of those who, with plentiful supplies of food urge the adoption of less intensive systems, where use of fertilisers, herbicides and insecticides are being phased out. But such systems are not likely to become the model for world food production, leaving the majority involved in food provision to strive for intensive systems of livestock and crop production, which are more benign with respect to the environment. It is in this context, that the development and use of genetically modified (GM) crops could offer considerable potential.

Currently over 50 GM crops have been approved for use in different countries. These cover a wide range of plant species with a significant number of modified traits, and viewed against man's increasing needs for food, clothing, etc. offer significant advantages in providing greater crop security. Introduction of such crops could in the long-term lead to environmental advantages, where replacement of non-specific insecticides by the greater specificity of GM crop insect control systems could provide beneficial changes in the diversity of insects and other fauna. Reductions in chemical herbicide and insecticide use will reduce contamination of water courses, whilst minimal tillage systems, possible with GM crops, will result in improved soil structures and reduced soil erosion. Furthermore, reduction in insect, viral and fungal damage of crops will benefit consumers through the provision of higher quality food, whilst modifications to nutritional entities (e.g. vitamins and essential fatty acids) to enhance overall human health could be a distinct possibility. Finally, such changes are likely to bring sustained economic benefits to farmers and growers and other trades involved in the production of food. In 1998, over 28×10⁶ ha of GM crops were planted and harvested globally (James, 1998), mostly in North America.

There is growing concern however within the United Kingdom and Europe regarding safety issues over the introduction of genes into plant genomes using recombinant DNA technology. The intention of this paper is to review the procedures used to generate GM crops, together with consideration of the possible consequences of dietary exposure of man to modified plant DNA. The effects of feed processing on possible DNA fragmentation will be considered and

discussed in relation to the need for feed labelling and concomitant issues associated with the consumption of food products derived from animals fed GM crops. Initially the paper will consider regulatory procedures that are currently in place with regard to the possible introduction of GM crops, along with a brief review of some of the factors which have heightened the concerns of the UK and European public regarding food safety.

Issues of food safety

Whilst Europe has raised many issues regarding GM crops, undoubtedly the major concern relates to the safety of food derived directly or indirectly from such crops. Of particular concern is the need for reassurance regarding the fate of DNA and resultant proteins derived from introduced traits. This has led to several important questions; (1) could the DNA of inserted or modified genes, or their products, if transferred to animals, cause adverse health effects in these animals; (2) could these DNA fragments or proteins be transferred to and accumulate in the products (milk, meat, eggs) of animals fed GM crops and (3) will consumption of agricultural crop materials or animal products derived from GM crops lead to adverse health effects in humans. This concern is understandable given recent experiences with bovine spongiform encephalopathy (BSE), *E. coli* 0157 in meat products and *Salmonella* in poultry, which have heightened the awareness of the European, and especially, the UK public. Consumers now demand greater food traceability, and if not satisfied with the information provided, their response is to call for the offending food to be banned. Thus hormone-implanted beef (as produced in the USA but no longer in the UK) is not wanted, yet it is only 12 years since this production practice was banned in the UK, and prior to that consumers voiced few concerns. A similar attitude prevails toward GM crops, yet products based on GM tomatoes and GM soya have been available and enjoyed within the UK for several years. For several reasons, the mood of the consumer is shifting towards sustainable systems, including free-range chickens, albeit these may be more disease compromised than their housed counterparts, and organically grown foods, where consistency of the product may never be realised. In summary, the UK consumer is often fickle with respect to choices and concerns, and easily swayed by 'public opinion'. This is most likely due to a lack of understanding of the often complex technologies involved, as well as a lack of unbiased and independent information, and has become a major issue facing growers who are committed to producing quality food at affordable prices, but with acceptable and proven technologies. Without the

introduction of GM crops, current levels of insecticide and herbicide use will continue, with little prospect of the erosion of the environment being abated. In this respect, biological rather than chemical control of unwanted weeds and insects represents a potential revolution in the production of crops for human use, provided safety of the food can be guaranteed.

Regulatory climate

Since first suggested, all GM crops have been subjected to rigorous and robust safety assessments by the Governments of the UK, EU and USA and other such organisations. However, this has not been sufficient to curb demands by some to either limit or totally ban the use or even testing of GM crops. Current regulatory directives embrace the need to provide an assessment of ecological risk, as well as testing of food safety and provision of suitable labelling to enable consumer choice. Within the UK, this responsibility resides with The Department of Environment, Transportation and the Regions (DETR), in conjunction with the Advisory Committee on Release to the Environment (ACRE), supported by the Ministry of Agriculture, Fisheries and Food (MAFF). All recommendations are then submitted to the European Commission. Meanwhile, products from GM crops are regulated by the Novel Foods regulation, which is under the jurisdiction of MAFF, with advice from the Advisory Committee on Novel Foods and Processes (ACNFP). Similarly all recommendations from MAFF regarding GM derived products are passed to the European Commission for further consideration.

To grow a GM crop in any EU country requires specific criteria to be met prior to planting, with additional tests performed during restricted limited acreage trials prior to possible commercialisation. Considerable precautions are taken to provide segregation of GM crops from conventional crops and wild relatives, in order to prevent potential escape of the GM crop through cross pollination with non-GM neighbours, (Masood, 1998). Subsequently, all novel crops are tested for food safety according to suitable regulations which have been developed. It is further recognised that GM feeds destined for animals must be tested, and suitable legislation is currently being developed. All these criteria have been established through full consultation with recognised safety experts from several industrialised countries. Recently the Royal Society published a review of the safety of GM crops for food use (1998), in which its expert committee examined;

'various aspects of the controversy, including the scientific evidence concerning the risk of transfer of genes from the GM crop plants to wild species and non-GM crops, the uptake of genes from GM crops by the digestive system and the current state of the regulatory system. The experts concluded that the chances of gene transfer happening are slight provided that the regulatory processes are followed, but that this must be kept under consideration'.

The committee also noted that MAFF is currently supporting research to examine the possible transfer of DNA from GM crop derived animal feed to animals and gut microbes, adding that quantification of such events and estimation of probability will be inherently difficult to establish.

Contemporary food safety issues in the UK

Undoubtedly the current concern over GM foods in the UK is related to other food scares experienced over recent years. Identification of stable prion particles as the causative agent of BSE, and *E. coli* strain 0157 as one of the most virulent food spoilage organisms known to man have alarmed the UK public and heightened consumer awareness of food safety, (Hillerton, 1998). In both cases, the agents for such diseases were derived from infected animals and their incidence can be traced to a number of questionable practices, including changing of the 'Protein feedstuff' regulations in the mid 1980's, compromised on-farm animal welfare and management, poor slaughterhouse sanitation, or sub-standard food preparation by processors and retailers. Concerns over GM crops do not fit into such

categories, but this has not been sufficient to allay the fears of the UK public, most of whom are unaware of the scientific issues.

Bovine spongiform encephalopathy and Creutzfeldt-Jacob disease

It is claimed that BSE, a neurological disease of cattle was transmitted by the use of compound feeds containing contaminated meat and bone meal (MBM). The infectious agent is a prion, which unlike bacterial or viral pathogens contains no DNA but instead is a single protein. The disease was identified in the UK in 1986, and by late 1987 was becoming an epidemic. Despite some initiatives from MAFF, the feeding of ruminant-derived MBM to ruminant livestock was not banned until 1988, and to non-ruminants in 1990, followed in 1991 by the banning of all MBM products, irrespective of source. It is claimed that during this time significant contamination of ruminant feeds due to cross-milling may have occurred. The overall contributory factor which eventually led to 1.7×10^5 cattle being infected, and a further 1×10^6 animals being slaughtered in a compulsory cohort cull during 1997/98, has been related to the introduction of new processing methods for the production of MBM, based on lower temperatures and reduced solvent extraction. Earlier rendering procedures were based on higher temperatures and increased solvent usage and appear to have been more effective, given that MBM had been used widely without problems for over 40 years.

To date, consumption of infected animal tissue (brain, thymus, spleen, spinal chord) is believed to be responsible for the death of approximately 40 adults in the UK from the human form of BSE, known as 'new variant Creutzfeldt Jakob Disease' (nvCJD), with reports of one death in France. The BSE prion is an extremely unusual variant of a naturally occurring neural specific protein (PrP), and survives digestion in the stomach, to be absorbed through the upper small intestine where it can affect cells of the lymphoreticular and central nervous systems. It is suggested that whilst nvCJD requires prior infection with a prion, susceptibility to the infectious disease may be inherited, (Hillerton, 1998). Other forms of CJD which are not caused by an infective prion also occur in man, but should not be confused with nvCJD.

E. coli, strain 0157:H7

The death of 20 people during an outbreak of food poisoning in Scotland in 1997 was linked to the consumption of inadequately prepared meat contaminated with a pathogenic strain of *E. coli* bacteria found on farms and in slaughterhouses (Williams, 1998). Other studies have demonstrated the occurrence of this bacteria in apparently healthy animals, birds, and insects, and in the water supply on farms with sick animals, (Shere *et al.*, 1998; Hancock *et al.*, 1998).

These and other issues including salmonella in poultry meat and eggs have reduced public confidence in the attitudes and actions of regulatory bodies with regard to the implementation of food safety and associated sanitary procedures. It is easy therefore to recognise why this lack of confidence has been extended to regulations governing the growing and release of GM crops to the environment. Many people feel, often on the basis of little or no evidence with scientific credibility that the introduced traits (i.e. DNA) and/or the resulting proteins in modified crops will lead to undesirable effects in humans. As well as direct exposure to GM crops, man's indirect exposure through possible accumulation of novel DNA or proteins in animal products is of concern whilst exposure of the animals per se is often raised as an issue.

Genes and genomes and DNA biochemistry

Deoxyribonucleic acid (DNA) provides the genetic coding for all plants, animals, bacteria and many viruses. In plants and animals it exists as a long molecule, basically one per chromosome, comprising of many ($>10^6$) small molecular units or nucleotides. Each nucleotide contains a pentose sugar, a phosphate group and one nitrogen-containing base (adenine, cytosine, guanine or thymine). Nucleotides are arranged in specific sequences, and matched by complimentary nucleotides on the opposite strand of the anti-parallel double helical

molecule, held together by hydrogen bonds. Linear groups of 1000 or more nucleotides act together as a functional unit known as genes. Each gene provides the blueprint for the production of specific proteins, whilst some genes contain regulatory sequence segments which do not code for proteins, but control specific functions including the spatial, temporal and quantitative expression patterns of genes throughout the lifetime of the organism. An average plant species contains between 20 and 50×10^6 different genes.

Many plants are diploid, having one homologous copy of each chromosome from each parent. Equally crop plants can be multiploid, containing four (eg soyabean with 2 diploid sets of chromosome resulting from complete duplication of one genome; autotetraploid) or six (eg wheat, with 3 complete sets of homologous chromosomes derived from different ancestral species; allohexaploid) haploid genomes. Polyploidy in cultivated crops is associated with agronomic vigour leading to increased yields. The haploid sequence of soyabeans was estimated by Clark (1997) to contain 1.1×10^9 bp, equivalent to the DNA content of the pollen or the primary nucleus of the ovule. The maize haploid genome is approximately 2.5×10^6 bp whilst the human genome is slightly larger (3×10^6 bp). In addition to the nuclear genome, two subcellular organelles (mitochondria and chloroplasts) contain small plasmid-like chromosomes with additional genes.

When cells replicate, the chromosomes are duplicated in a process that copies the genes with high fidelity. Few changes or mutations occur normally during the duplication process, whilst any that do are usually corrected during the synthesis phase of mitosis. Most mutations are inconsequential with respect to non-coding DNA, whilst those occurring within the coding region are generally neutral but occasionally can be deleterious. On the other hand, a small percentage of mutations will code for functionally improved proteins. Such mutations are desirable as they provide genetic diversity essential for the organism to adapt to changing environments. A second type of mutation is the addition or deletion of segments of the chromosome of sufficient size to code for a complete protein or a group of proteins. Examples of this are seen with some viruses which insert DNA into the chromosomes of their natural hosts, whilst there are specific circular plasmids from bacteria (eg Ti plasmid from *Agrobacterium tumefaciens*) that can similarly insert DNA segments into the genome of the infected plant. This has now become one of the primary tools used in the genetic modification of plants (Zambryski, 1992).

The genetic modification process

Naked segments of DNA are not normally taken into mammalian cells except by terminally differentiated macrophages that routinely phagocytize cell fragments including DNA. Consequently, to achieve targeted genetic changes in plants, highly specialised techniques have been developed, including inoculation of plants with bacteria containing Ti plasmids with specific gene inserts. Alternatively, specific DNA fragments can be inserted into cells using chemical or physical means (eg DNA coated gold particles projected with sonic pressure). Using such methods it is possible to produce GM plants through the introduction of DNA into the plant chromosome at a random site by specific enzymes. Success depends upon the introduced DNA becoming stably integrated into one of the chromosomes, with this DNA being passed to new plant generations through conventional breeding procedures. Once integrated and duplicated, the molecular characteristics of the transferred DNA segment will be identical to the original plant DNA.

The size or length of DNA inserted into the genome of an alternative organism will vary, but is usually of the order of 2 to 14×10^6 bp, examples being 4×10^6 for insect-resistant maize and 2.5×10^6 for herbicide-tolerant soyabean. The haploid genome size of these crops is approximately 1×10^9 bp, with the inserted DNA constituting less than 0.00016 to 0.00066% of the genomic DNA in the modified plant.

Safety evaluations of GM crops

Regulatory agencies in Europe, USA, Canada, Japan, and other countries require all GM crops to be subjected to extensive safety trials and field trial evaluation before being released for Agricultural use. Such procedures can take between 7 to 10 years before final commercialisation of new GM crops.

Elaborating on the procedures involved, all agencies call for extensive characterisation and safety evaluation of the novel protein resulting from the inserted gene. Protein accumulation levels, as evidence of gene expression in plant tissues are routinely measured, taking account of the different morphological parts of the plant (leaves, stems and roots). Selection of potentially useful GM crops for further exploitation includes evaluation of productive growth, with respect to the development of leaves, stems, flowers and grain or fruit. This will ensure that DNA insertion has not disrupted any genes critical to normal plant development or altered the expression of important genes. The agronomic characteristics of the plant are tested along with full monitoring for stability of gene inheritance.

Protein safety

To provide evidence of safety with respect to introduced protein, the first step is to purify the protein followed by amino acid sequencing, molecular weight determination and assessment of solubility and stability. Protein digestibility studies are normally undertaken and where the protein has been produced from recombinant microbes, bridging studies are conducted to ensure that the plant protein is equivalent to the protein produced by the microbes. Subject to the acquisition of satisfactory data, the next stage would be to conduct acute toxicity studies by oral administration of the protein to mice at doses many times higher than the levels which humans would be expected to receive when consuming the GM crop. Changes in animal body weights would be monitored for a minimum of 14 days along with clinical observations, before the animals are euthanised, followed by necropsies to examine major organs. Any adverse findings during this phase, including poor weight gain or abnormal appearance of tissues and organs would either lead to more testing or possible abandonment of the specific line of development.

In studies which include animals, or possibly at a later stage humans, one concern is to establish whether the test subjects have been previously exposed to the novel protein. The herbicide tolerant gene in GM soya and maize is similar to the naturally occurring maize EPSP synthase which has been consumed safely ever since animals and man first started to consume maize. Equally several insect resistant proteins isolated from *Bacillus sp.* are virtually identical to proteins in the bacteria, *Bacillus thuringiensis*, which have been identified as the active agent in the control of insects on organic farms, a practice which has occurred without problem for the last 40 or so years.

Substantial equivalence and animal wholesomeness

Plant tissues from GM crops are routinely analysed for most major nutrients including total protein and lipid, plus possible anti-nutritional factors and other specific attributes of the crop. This has been referred to as 'substantial equivalence' by FAO/WHO. The data are then compared with that from non-GM varieties of the same crop, whilst GM crops which may be used in animal feeds or consumed by wildlife are subjected to animal wholesomeness studies. In these GM crop materials are prepared and fed to sensitive animal species with studies to date involving broiler chickens, trout, catfish, goats and most farm animal species. Proteins introduced to confer insect resistance, such as those derived from *Bacillus thuringiensis* are tested to establish if they are toxic to non-target organisms including ladybirds, bees and soil detritivores. The precise details of the studies will vary with different crops but all are reviewed by the regulatory authorities prior to study initiation and upon submission of the data. The primary objectives of these studies are to establish that the GM crops are nutritionally equivalent to unmodified commercial varieties and that the introduced proteins are safe to humans, animals and the environment.

Consumption of DNA

Both the World Health Organisation (1993) and the US Food and Drug Administration (1992) have previously concluded that there is no inherent risk in consuming DNA, including that derived from GM crops. The basis of their conclusion was that mammals have always consumed significant quantities of DNA from a wide variety of sources, including plants, animals, bacteria, parasites and viruses.

Dietary exposure to foreign DNA

The obvious route for human and animal exposure to foreign DNA is by oral consumption. Most foodstuffs contain a complex mixture of proteins, lipids, carbohydrates, nucleic acids, minerals and vitamins. The relative proportions of these may vary widely, but the quantity of DNA in most food crops is generally less than 0.02% (dry matter basis), with Watson and Thompson (1988) reporting values of 0.005% in some crops. At such low levels of abundance it is difficult to provide realistic estimates of DNA intake for typical human diets, whilst nutritional studies have demonstrated that most of the DNA is enzymatically degraded in the alimentary tract, usually prior to the small intestine. Once DNA is fragmented its functionality will be lost and often even its source can not be identified. It is pertinent to note that other sources will contribute to DNA in gut contents, including shed epithelial cells and white blood cells, along with bacteria and protozoa resident in the gut. To calculate maximum possible exposure to GM-derived DNA it must be assumed that no degradation of DNA occurred in the gut following ingestion. This permits estimation of the maximum possible intake of recombinant DNA from GM crops in relation to total DNA intake, taking into account the proportion of total DNA to total feed intake, and the proportion of each commodity in the diet. Consider the impact of GM maize fed to dairy cows either as forage maize silage or as maize grain. Based on maize silage and maize grain accounting for 40% and 20% respectively of total dry matter intake, (i.e. 60% of total ration), in a 600 kg dairy cow, transgene DNA consumption would amount to 2.6 µg/day according to;

$$\left(\frac{4000 \text{ bp insert DNA}}{2.5 \times 10^9 \text{ bp genomic DNA}} \right) \times 0.0001 \text{ g DNA/g DM} \times 0.60 \text{ g maize/g DM} \times 24000 \text{ g feed/day}$$

This compares with a total diet DNA intake of 608 mg/day, equating to a GM DNA to normal plant DNA ratio of 1:234,000 or 0.00042% of total dietary DNA. On this basis it appears that exposure to introduced DNA of GM crop material will be negligible compared with normal exposure to non-GM crop DNA.

In vivo fate of DNA

Comprehensive data relating to DNA exposure and possible fate can be derived from studies involving the analysis of human and animal milk, designed to estimate the dietary requirements of nucleotides (as phosphate esters of nucleosides; adenosine, guanosine, thymidine, cytidine, uridine; as free molecules and as components of DNA and RNA) for suckling neonates (Gil and Uauy, 1995). As nucleotides are generally abundant in food relative to dietary requirements, little attention has been paid to the occurrence of DNA and RNA in food (Jackson *et al.*, 1997; Yu, 1998). Ingested DNA is rapidly cleaved into small fragments by the mechanical processes of mastication along with buccal and gastro-intestinal enzymatic digestion and acid hydrolysis. Few studies have attempted to measure *in vivo* DNA degradation and published evaluations occurred prior to the development of analytical methods that could distinguish the source of DNA (plant, digestive tract microbes, epithelial cells etc). In particular the methods could not determine the length of the DNA fragments but were able to establish the catabolism of DNA to nitrogenous bases, free bases and secondary metabolites (McAllan, 1980). The enzymes involved in DNA hydrolysis include high concentrations of DNase I, an endonuclease that disrupts the double stranded DNA and is produced and secreted by the salivary glands, as well as the pancreas, the liver and the Paneth cells of the small intestine. DNase I has optimal activity at neutral pH. DNase II is a more recently characterised

enzyme (Baker *et al.*, 1998; Yamanka *et al.*, 1974) and has a pH optima of between 4.6 and 5.5. This enzyme is also secreted but its primary function is in lysosomes within phagocytes, involved in the catabolism of DNA as well as the fragmentation of genomic DNA during apoptosis. DNase II causes double strand breaks in native DNA, by first nicking one strand followed by the other at approximately the same point (Tsubota *et al.*, 1974). Although the *in vivo* enzymatic reactions of DNases are not well characterised, in *in vitro* studies, DNase I and II have been shown to cleave both single and double stranded DNA primarily at the 3' end of pyrimidines, most likely between nucleosomal structures and along stretches of naked double stranded DNA. Random sized pieces of blunt end DNA fragments are produced by these reactions. McAllan, (1982) estimated that more than 85% of the plant DNA consumed by ruminants is reduced to nucleotides or smaller constituents before entering the duodenum, with most of the larger nucleic acid fragments in small intestinal contents arising from rumen microbes.

In addition to enzymatic digestion, low pH conditions in stomach (monogastrics) or abomasal (ruminants) contents should remove most adenine and guanine bases from naked DNA fragments. This process will destroy the genetic information in long strands of DNA, similar to what occurs following *in vitro* depurination (Klinedinst and Drinkwater, 1992). However studies on gastric depurination reactions and kinetics have not been reported.

DNA transfer

A small proportion of plant or microbial DNA fragments remaining in intestinal digesta could potentially be absorbed through the intestinal mucosa either directly by epithelial cells or by antigen presenting cells of the immune system. If the intestinal epithelial surface has been damaged, DNA and other macromolecules may also diffuse into the lamina propria. It is suggested however that most of this DNA would be phagocytised by tissue macrophages, dendritic cells or other terminally differentiated phagocytes of the immune system. Owing to technical difficulties associated with the measurement of absorbed plant DNA above normal high background levels of endogenous DNA derived from the animals cellular DNA as well as ruminal and intestinal microbes, few studies have been reported on this aspect. In one recent study however, Klotz and Einspanier (1998), reported the detection of a plant DNA fragment in tissues taken from a cow. The fragment was from the abundant soyabean chloroplast gene (ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) large subunit) and was detected in white blood cells of a cow fed a diet containing GM soyabean meal. The reported assay used the highly sensitive polymerase chain reaction (PCR) method followed by Southern blotting to detect the amplified DNA fragment. In the same experiment, attempts to detect the presence of DNA from the GM transgene CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) in cows blood were unsuccessful, despite being detectable in the soyabean meal. Equally they could not detect fragments of Rubisco or EPSP synthase DNA in milk collected from that cow.

In another study, microbial DNA was fed directly into the gastrointestinal lumen and tissues of mice and fragments of this DNA were detected in some mouse white blood cells (leukocytes) at 24 hours or more after initial exposure (Schubbert *et al.*, 1994, 1997, 1998; Doerfler *et al.*, 1997; Doerfler and Schubbert, 1998). Whilst the elegance of this research is not questioned, there are a number of important factors to consider when judging whether the findings can be generalised as relevant to normal dietary patterns of consumption.

Methylation of tested DNA

Bacteriophage M13 DNA was used in these studies and the equivalence of this to DNA derived from plant material needs to be questioned. The bacteriophage M13mp18 and plasmid pEGFP-C1 DNA used was produced in *E. coli* bacteria and was either closed circular (native) or linearised by digesting with a restriction enzyme. The M13 genome is 7250 bp and contains 29,5'-purpurCpGpyrpyr-3' (purine, cytosine, guanine pyrimidine, pyrimide) nucleotide

sequences in which the cytosine would not be methylated when produced in *E. coli*. The plasmid pEGFP-C1 used in some experiments contains 16,5'-purpurCpGpyrpyr-3' sequences in 4371 bp and would also not be methylated when synthesised in *E. coli*. In contrast it is expected that these sequences would be methylated within normal plant and animal cells. Plant cytosine DNA methyltransferases as identified in *Arabidopsis thaliana* and *Triticum* sp. (Vlasova and Vanyushin, 1998) would methylate the cytosine within these sequences in a manner similar to the pattern identified in mammalian DNA. These enzymes occur in both mono- and di-cotyledonous plants and are assumed to be generally present in most flowering plants.

The significance of unmethylated microbial DNA is that such sequences have been shown to markedly upregulate inflammatory cell activity and stimulate a vigorous immune response. (Yi and Kreig, 1998; Klinman *et al.*, 1997; Sato *et al.*, 1996). Mutations of the 5'-purpurCpGpyrpyr-3' sequence in bacterial plasmids or synthetic methylation of the sequence significantly reduces the ability of DNA containing the sequence to stimulate a T cell response (Sato *et al.*, 1996). Mucosal surfaces, particularly in the respiratory tract and upper intestine contain significant primary antigen presenting cells, dendritic cells, macrophages and B lymphocytes. All can engulf DNA either through pinocytosis or phagocytosis. Once activated, dendritic cells migrate to lymphoid tissue to specifically engage and present antigen to naive T and B lymphocytes in order to induce antigen specific immune responses. Following activation, enhanced cellular functions include non-specific endocytosis (uptake) and an increase in antigen processing and presentation within endosomal vesicles (Hacker *et al.*, 1998). The occurrence of these sequences in the studies of Doerfleuer and Schubbert may well account for the relatively common finding of microbial DNA in white blood cells of various tissues. On the other side of this, methylation of DNA often reduces the amount of depurination and degradation that can occur, and so might result in larger, more intact fragments entering the system. However, for the persistence of exogenous DNA to be of any real significance, it must either disrupt normal host gene function (which is discussed later) or the entire gene must be inserted into the host genome in such a way as to allow transcription and then expression of the resulting protein.

Potential trans-placental passage of M13 DNA

Studies involving fluorescent *in situ* hybridisation (FISH) probing of mouse tissues and cells demonstrated signals associated with chromosomes in a few cells of the M13 fed mice (Schubbert *et al.*, 1997, 1998). It was suggested that these provided evidence that fragments of DNA from M13 had integrated into the chromosomes of somatic cells in mice given the DNA by oral gavage or in feed. A similar approach was used to identify cells carrying M13 DNA in foetal and new born offspring of M13 DNA dosed female mice. However hybridisation at identical positions on two chromatids of the same chromosome which would suggest integration and replication of the inserted gene was only observed in one cell, (Schubbert *et al.*, 1998). These authors noted that it was the only cell exhibiting that pattern in the animal and concluded that naked DNA from the serum of the mother had crossed the placenta and been taken into a foetal somatic cell.

An alternative explanation to this however may be that white blood cells carrying M13 DNA fragments crossed the placental barrier and entered the foetus. There are a few reports of maternal blood cells in the umbilical cord of human foetuses during the third trimester whilst two studies have demonstrated the existence of maternal cells in foetal blood during the second trimester, (Petit *et al.*, 1997; Lo *et al.*, 1998). It is therefore possible that leukocytes of the dam phagocytosed the DNA before crossing the placenta, to enter the circulatory system and ultimately foetal tissues. However regardless of whether the M13 DNA containing cells were of maternal or foetal origin, they were most probably leukocytes. Whilst currently there is no direct evidence that non-methylated CpG DNA of M13 would stimulate

foetal phagocytes, published data suggest this may be a distinct possibility.

Leukocyte phagocytosis of microbial DNA

Data from a number of DNA vaccine trials with humans and other species indicate that cells take up native DNA with very low efficiency unless there is an adjuvant such as unmethylated CpG DNA, liposomes, polyamines or other such stimulatory molecules associated with the DNA. As reported by Schubbert *et al.* (1997) most of the cells containing M13 DNA fragments were leukocytes. Since activation of leukocytes is more stimulated by unmethylated CpG sequences, the observations probably represent a normal response to microbial DNA that would not be expected to occur with plant DNA. Thus it is suggested that their experiments did not provide a satisfactory estimate of the rate of absorption or cellular uptake of plant DNA from the mammalian digestive tract.

Analytical uncertainties

Outwith of these concerns, consideration must be given to the analytical techniques used. Studies by Doerfleuer and Schubbert utilised both PCR and *in situ* hybridisation to measure fragments of test DNA absorbed from the digestive tract and taken up by cells. None of the studies established detection of the test DNA (either M13 or plasmid) in all animals or all tissues, with the various studies indicating positive detection in 20 to 90% of the mice dosed. One possible interpretation for this finding is that sample concentration of test DNA was close to the limits of the detection methods used.

To evaluate the possible limit of detection of plant DNA in a cow relative to that determined in a mouse model, it would be necessary to compare the dose of a single copy foreign gene to both animals on an equimolar basis. From Doerfleuer and Schubbert's studies, it can be estimated that the total quantity of DNA given as a single oral dose is approximately proportional to the total amount of maize DNA that a normal cow could eat in 24 h. However the relative concentration of the detectable specific single gene is markedly higher in the mouse than in the cow. In mid lactation, a cow may consume up to 600 mg of maize DNA per day. The maize diploid genome is approximately 5×10^9 bp and since the DNA insert size in GM maize is 4000 bp (one copy per diploid genome), the relative amount of transgenic DNA fed to the cow is $0.48 \mu\text{g} \left(\frac{4000}{5 \times 10^9} \right) \times 600 \text{ mg}$. This should then be compared with $50 \mu\text{g}$ of M13 DNA fed to the mouse, a ratio of 104:1 (mouse to cow).

A further factor relating to detection levels is the ratio of samples to body weight, and the assumption of a uniform distribution of DNA in both animals. The ratio of mouse to cow body weight is approximately 1:24,000. The relative concentration of detectable DNA in the mouse compared with the cow would be equal to the relative amount of DNA divided by the body weight ratio, or 2.53×10^6 . As a consequence, the method of detection required to measure a specific fragment of maize DNA, including the transgene in the tissues of a cow would have to be 2.53×10^6 times more sensitive than those used to detect M13 in mouse experiments if similar results are to be obtained. This is on the assumption that the rate of uptake and retention of plant DNA in the cow was similar to that of M13 in mice. Based on the ratio of molar differences in plant DNA to M13 indicated above, any PCR assay designed to detect plant DNA in cow tissues would require an additional 21 cycles of amplification, assuming 100% efficiency of amplification, for the same limit of detection. The other method used to detect M13 DNA in mouse, namely *in situ* hybridisation, would not be expected to yield any positive results in the cow using a probe for a single gene like EPSP synthase as only a few cells were identified in the mouse with M13 as the target DNA.

Evidence from the Doerfleuer and Schubbert group established that a minute quantity of unmethylated microbial DNA may be transferred from the digestive tract of animals into white blood cells, and possibly mammalian cells. While the DNA being transferred into plant cells in order to produce a GM crop is from bacteria and any 5'-pur-

purCpGpyrpyr-3' sequences within the transferred DNA would not be methylated when transferred (Jones, 1999), the process of replication of the DNA within the plant would cause it to be methylated and thus become similar to normal plant DNA. Therefore, the transferred DNA will not be immuno-stimulatory as is bacterial DNA. For that reason, in our estimation, the frequency of plant DNA being taken into cells is probably much lower than indicated by the studies which used unmethylated model DNA (M13 or plasmid DNA) as the test substance. Additionally all of the reported investigations have focussed on the presence of fragments of DNA rather than entire genes, which would clearly have been more significant.

Potential cloning of inserted ingested DNA

The reported incidence of positive signals from prepared leukocytes within the first few hours after dosing was approximately 1:1,000 cells (Schubbert *et al.*, 1997). These authors reported cloning and sequences of four separate clones containing DNA segments which included a fragment of M13 DNA fused to another DNA. These clones were obtained from screening a total of 1.25×10^8 clones from three genomic lambda phage libraries produced using DNA from the livers of mice fed M13 DNA (Schubbert *et al.*, 1997, 1998). A fifth clone contained only M13 DNA. In two of these fusion clones, the non-M13 sequence segments were short pieces of *E. coli* DNA. The other two clones contained short segments of DNA representing probable mouse pseudogenes, one with 70% homology to the mouse IgE receptor gene, and the other with 81% homology to the mouse beta-2-thyroid hormone receptor gene, fused to fragments of M13 DNA. The clone containing the fragment of the IgE receptor pseudogene was discussed by Schubbert *et al.* (1997 and 1998) and Doerfler *et al.* (1997). While it is possible that these clones represent foreign DNA inserted into the mouse genome, it is also possible that they were cloning artefacts produced during the ligation reactions with the lambda phage DNA during construction of the genomic libraries. It could be argued that the two fusion clones with genes from *E. coli* fused to M13 are evidence that these are likely to be artefacts.

In conclusion, it would be difficult and probably not valid to extrapolate the findings of the Doerfler-Schubbert group from the uptake of bacteriophage and plasmid DNA in mice to predict the likelihood that plant DNA from either GM or non-GM crops would be absorbed and integrated into livestock or humans.

Implications of the uptake and integration of foreign DNA

Assuming all consumed plant DNA can be absorbed and inserted into the chromosomes of some cells, one possible outcome is that the novel protein may be expressed in a mammalian cell. This assumes that the novel DNA was inserted in a transcriptionally active region of the chromosome, in juxtaposition with, or contained an appropriate promoter, transcriptional site, ribosomal binding site and stretch of translatable protein coding sequence. Since the transgenes of the GM crop would be only one or a few additional genes in the 20,000 to 50,000 genes of the plant genome, the most probable outcome is that one of the other plant genes would have been inserted and expressed. Currently there is no evidence that any plant proteins are expressed in tissues of any animals that have consumed plant material. Indeed, no plant gene (or fragment of) has ever been detected in the human genome or that of any other animal. Since this is the result of millions of years evolution with constant exposure to plant DNA, the unaided integration of a GM plant gene is highly unlikely. Even if the transgene were to be expressed in an animal, there should still not be any concerns. The transgene protein products will have been tested in an acute mouse toxicity assay and extensively evaluated for safety prior to commercial release of the product or the seed. The cells that are most likely to have internalised the plant DNA are non-reproducing and terminally differentiated (eg leukocytes). Expression of any of the plant proteins would be thus be limited to the life of that specific cell. None of these scenarios suggest possible harmful events arising from expression of a protein from DNA which might be incorporated into cellular DNA.

If any DNA fragment were inserted in the middle of the coding region of an expressed mammalian gene the most likely consequence is that the endogenous gene product, the protein, would be non-functional. The insertion may interrupt the coding region or alter expression by interfering with a native promoter or enhancer element. As mammals are diploid, the other copy of any affected gene would probably still function to produce the endogenous gene product.

In this respect, Doerfler *et al.* (1997) reviewed the potential events that could occur with the possible integration of DNA fragments into the mammalian genome including interruption of genes, activation of oncogenes and alteration to promoter regions with subsequent alteration of normal expression. They speculated that the mechanism for altered expression following integration is likely to be hypermethylation, and consequently the attenuation of adjacent genes. Schubbert *et al.* (1998) noted that it will be very challenging to investigate the possible consequences of insertion of non-viral foreign DNA into genomes of recipient organisms. While their assessment of some possible consequences is plausible, they did not demonstrate any of these events nor did they demonstrate any ill effects in mice exposed to M13 or plasmid DNA. Their suggestions of potential consequences should therefore remain as speculative.

Since it is now believed that most cancers occur after one or more mutation within a single cell, the chance that single insertion of DNA anywhere in the genome of an animal would cause cancer is extremely small. Searches of the literature failed to identify any publications which predicted the probability of cancer development from the insertion of non-viral DNA from any possible exposure including ingestion. However there are some risk assessments for DNA vaccines, including Kurth (1995) who predicted an incidence of one cancer from 10×10^6 intramuscular vaccinations with 10 µg DNA. The hypothesis for the mechanism is by activation of proto-oncogenes via insertion of the DNA proximate to the promoter. Since absorption of DNA from the digestive tract is extremely inefficient, especially when compared with direct injection of DNA, the probability of cancer occurring by ingestion of DNA is clearly much reduced compared with vaccination. Additionally, the risk associated with the transgene of a GM crop is no greater than the risk associated with the insertion of any fragment from the original plant genome DNA. Since plant genomic DNA is present at more than 100,000 times the concentration of the transgene, the additional risk by having the transgene in the plant is negligible relative to the risk of acquiring genes from the non GM material. However DNA of viral origin is often more 'mobile' in its ability to integrate and subvert the host genome; one of the primary reasons attenuated or modified viruses are often used as a delivery and targeting system for the creation of GM organisms. Whilst this does not increase the risk of GM crops to humans or animals, it does raise the possibility of increasing the chance of transfer to other plant species.

DNA integrity with feed processing

It is recognised that certain processes involved in animal feed preparation will cause significant fragmentation of the DNA, whilst other processes appear to have little or no effect. In a preliminary report of MAFF sponsored research, Forbes *et al.* (1998) provided data on DNA fragment size from various processed feed fractions of wheat, maize, soyabean, linseed, rape seed, sugar beet and rye grass. Treatments involved in the preparation of feed material include variations in mechanical processing, heating in dry air or with steam at different pressures and possible cubing/extrusion of the final product. DNA fragments were analysed by electrophoresis of sample extracts on agar gels which were stained with ethidium bromide and viewed under UV illumination to compare the size range of the DNA smears with marker standards. Additionally, maize samples were analysed using PCR with primers specific for a 600 bp segment of the ribulose-1,5-biphosphate carboxylase/oxygenase small sub-unit gene situated in the nucleus. Results indicated grinding and milling to have little effect on DNA fragment size, whilst mechanical expulsion of oil

from seeds, or chemical extraction caused extensive DNA fragmentation. Dry heat applied to plant material at 90°C appeared to have no effect, while 95°C for 5 minutes caused considerable fragmentation of the plant DNA. Equally steam heat at low to moderate pressures effected substantial DNA fragmentation, whilst ensiling of forage had no detectable effect.

Antibiotic resistance marker DNA

In a recent publication Forbes (1998) suggested that feeding GM plants containing genomic antibiotic resistant marker genes could lead to the development of new antibiotic resistant microbes. Transmission of antibiotic resistance among bacterial strains is well documented, and occurs by transfer of plasmids, small circular extra-chromosomal pieces of DNA or in some bacterial species by insertion of intact antibiotic resistant genes from genomic DNA of one bacteria to that of another. However, there is only one species of bacteria (*Acinetobacter* sp. BD413) that has been shown to have incorporated a fragment of plant DNA. This bacterium is highly unusual in being naturally competent to be transformed with linear DNA from the environment. It has been shown that plant DNA containing the npt II gene, which encodes resistance to neomycin and kanamycin can at low frequency rescue *Acinetobacter* sp that already have an npt II gene containing a small deletion in the gene (Gebhard and Smalla, 1998; de Vries and Wackernagel, 1998). It was concluded that a recombination–repair of the npt II gene occurred with a segment of the plant DNA being spliced into the defective bacterial gene at a frequency of approximately 5×10^{-9} . However these studies did not demonstrate the uptake and function of a complete plant npt II gene, which suggests that de novo acquisition of complete genes from plants is extremely unlikely even in the presence of antibiotics providing selection pressure for recombinants. Although *Acinetobacter* sp BD413 can be induced by nutrients to acquire competence under soil conditions (Nielsen *et al.* 1997) a study involving the field release of transgenic soya beet containing the npt II gene failed to demonstrate horizontal transfer of this gene from sugar beet to soil micro organisms (Gebhard and Smalla, 1999). Additionally, the extensive use of powerful antibiotics as often occurs in intensive animal farming, has resulted in multiple resistance microbes. This must be a far greater cause for concern than the potential for resistance to the antibiotics commonly used for selection during the genetic modification process.

Labelling

Labelling of GM food products to provide consumer choice is required in Europe, and already occurs under the present regulations relating to herbicide tolerant soyabean and maize. Suitable methods are available to test for the presence of the protein product or the inserted DNA of the GM crop. However, labelling may not be required for those foods which have been shown to contain no measurable amounts of novel protein or DNA, as verification of GM and conventional plant materials is not possible. Included in this category would be refined cooking oils from GM plants. It has also been recommended that threshold limits are established that would be set to designate the limits of which products would require labelling as GM commodities, with those foods containing less than the threshold level of GM product not requiring to be labelled.

When the question raised by consumers becomes one of choice regarding the consumption of meat, dairy or poultry products from animals that may or may not have consumed GM crop material, the relevance and the practicality of implementing and enforcing any labeling policy would be challenging. When livestock consume plant DNA and proteins, nearly, if not all of the protein and DNA from the plant will be digested and the identifiable characteristics of the GM material would be destroyed. Once that plant material passes the stomach or abomasum, it is unlikely that any of the transgenic protein product would be detectable. Because most of the GM transgenic proteins are expressed at low concentrations, it is often difficult to detect them when the concentration of the plant is less than one percent of the test matrix, included in the feed or processed food. A

thorough literature search has not identified any studies describing the detection of plant proteins which are expressed at levels comparable to current GM products, in products including meat, milk and eggs derived from animals consuming the plant. Furthermore, as indicated in the discussion above, there are no examples of the detection of the DNA from single copy plant genes in animal products even when highly sensitive PCR assays were employed. Therefore, any suggestion to label food products derived from animals fed GM plant material could be impractical or difficult to enforce. One solution would be to allow the marketing of food products derived from animals that were not fed GM feed to be labelled as such, as long as proof of appropriate assurance schemes established that they were fed GM crop free feed. That approach is similar to what is required for labelling food as being "organically grown".

Conclusions

Currently relevant questions are being asked about the safety of GM crops that are entering the food chain. There are established regulatory requirements that must be met before commercial release of each GM crop. Those requirements include extensive characterization, testing and assessment of the safety of food or feed derived from GM crops. Currently the European Union, USA, Japan, Australia, Canada, Argentina and a number of other countries have evaluation processes that demand a rigorous spectrum of safety tests before GM crops can be grown in, or imported into, these countries. Additionally there is a growing body of scientifically valid information available that indicates no significant risk associated with the consumption of DNA or the resulting proteins from the GM crops that are registered in any of these countries. Based on the safety analyses required for each crop, consumption of milk, meat and eggs produced from animals fed GM crops should be considered to be as safe as traditional practices.

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