16 Genetically Modified Foods: Potential Human Health Effects

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Introduction

The scope of this review is restricted to data-based considerations about the safety of genetically modified (GM) foods of plant origin for health. No opinions unless supported by experimental results will be discussed. The emphasis will be on papers published in peer-reviewed journals. A few articles will be mentioned from non-peer-reviewed journals but only if they influenced the development of science-based ideas for the regulatory process. Environmental issues will not be dealt with.

Safety evaluation of whole foods derived from crops with considerable natural variability is more difficult than that of a single chemical, pharmaceutical or food additive, or defined mixtures of them. Published results of tests for toxicity and nutritional wholesomeness of complex foodstuffs are therefore few and far between. A recent comment in *Science* described this in its title: 'Health risks of genetically modified foods: many opinions but few data' (Domingo, 2000). Even a cursory look at the list of references of a recent major review on food safety issues (Kuiper *et al*., 2001) showed that most of the publications referred to were non-peer-reviewed institutional opinions or envisaged future scientific and methodological developments for safety assessments, but were short on actual published scientific papers on which a reliable database of safety could be founded. Judging by the absence of published data in peer-reviewed scientific literature, apparently no human clinical trials with GM food have ever been conducted. Most attempts to establish the safety of GM food have been indirect. At best, inferences have been drawn from animal trials, but the preferred approach is to use compositional comparisons between the GM foodstuff and its traditional counterpart. If these results show no significant differences, the two foodstuffs are 'substantially equivalent', meaning that the GM food is as safe as the non-GM food. Thus, as the regulation is almost exclusively dependent on 'substantial equivalence', the published results of GM food analyses and inferences drawn from them for health will be examined critically in this review.

In genetic modification, the intended gene is incorporated into the genome of a crop using a vector containing several other genes, including, as a minimum, viral promoters, transcription terminators, antibiotic resistance marker genes and reporter genes. Although in GM food safety the role of the intended gene is very important, the potential

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effects of these other genes need also to be taken into account because other parts of the construct or the insertion of the vector could contribute substantially to the overall effect (Ewen and Pusztai, 1999a). There is in fact some evidence that some of the other genes of the vector may have an effect on safety. This is particularly so as it is now known that DNA does not always break down in the alimentary tract (Schubbert *et al*., 1994, 1998; Hohlweg and Doerfler, 2001). This opens up the possibility that the antibiotic resistance marker gene, in addition to others, may be taken up by bacteria in the digestive tract and contribute to the spreading of antibiotic resistance via human gut bacteria. In this context, one potentially important observation was that a substantial proportion (6–25%) of a genetically engineered plasmid survived a 1-h exposure to human saliva (Mercer *et al*., 1999). Partially degraded plasmid DNA also successfully transformed *Streptococcus gordonii*, a bacterium that normally lives in the human mouth. Saliva also contains factors which increase the ability of bacteria to become transformed by naked DNA. Therefore, the prospect of the uptake of undegraded or partially degraded vector genes, including the antibiotic resistance gene, will have to be seriously considered. However, the main concern in GM food safety is what are the direct effects of the expression of the main intended gene after its insertion into the plant genome via a gene construct. An additional concern is that this may also cause significant, indirect and unintended effects on the expression and functionality of the plant's own genes. The number of copies of the construct inserted and their location in the plant genome (pleiotropic effect) are of particular importance in this respect, with the possibility that many unexpected changes may occur. This possibility is in fact generally accepted, and the inadequacy of the currently used methods to detect them is frequently acknowledged (Kuiper *et al*., 2001). Pleiotropic effects always occur with both conventional cross-breeding and genetic engineering, and their unwanted consequences usually are eliminated by empirically selecting for the desired trait and discarding the potentially harmful ones. Some of these changes are unpredictable and therefore we can only compare the known properties and constituents but cannot look for, or even less analyse, unknown components. This imposes limitations on our selection criteria. Reliance based solely on chemical analysis of macro/micronutrients and known toxins is at best inadequate and, at worst, dangerous. More sophisticated analytical methods need to be devised, such as mRNA fingerprinting, proteomics and secondary metabolite profiling (Kuiper *et al*., 1999). However, and most importantly, there is an urgent need to develop comprehensive toxicological/nutritional methods to screen for the unintended potentially deleterious consequences for human/animal health of genetic manipulation to pinpoint the problems in advance of the incorporation of the GM foodstuff into the food chain (Ewen and Pusztai, 1999b). Although some limited animal tests have been done, only a few of these have been published. However, data from some of these studies recently have been placed on the Internet. Although they were not peer reviewed, they were incorporated into this review because of their potential importance for other scientists.

Non-peer-reviewed Safety Tests on Commercial GM Crops in the Public Domain

FLAVR SAVR™ tomatoes

The first example of official safety evaluations of a GM crop, Calgene's FLAVR SAVR™ tomato, including a 28-day rat feeding trial, was commissioned by Calgene for the Food and Drug Administration (FDA) before its general release. Although the details of this study have never been published properly, because this work had such an extraordinarily major effect and influenced GM food regulation in the USA and elsewhere, there is a compelling need to analyse the methods used and the conclusions reached. Fortunately, as a result of a court case in the USA (Alliance for Bio-Integrity *et al*. vs. Shalala *et al*.), most data in the FDA's files are now on the Internet in the public

domain and can therefore be evaluated (Alliance for Bio-Integrity, 1998).

This GM tomato study shows most of the problems which may be encountered in GM food safety evaluation, particularly if, like the tomato, they are fruits rather than foodstuffs and their protein and energy contents are insufficient for supporting the growth of young animals. The methods used and results obtained in this study are important not only for their own sake but also for their influence on the process of regulation.

Substantial equivalence

As 'substantial equivalence' features so prominently in GM food regulation (Kuiper *et al*., 2001), including in this GM tomato study, there is a need to look more closely at this concept. This issue has been dealt with in some depth by a recent article (Millstone *et al*., 1999) in which the problems with this concept were highlighted, such as that 'substantial equivalence' has never been defined properly and that there are no legally binding rules on how to establish it in practice.

Differences in growth conditions can have a serious impact on composition and, therefore, in the absence of specification of the origin and the conditions of cultivation of the different GM and non-GM samples, strict scientific comparisons cannot be made. These are not valid unless the parent line is grown side by side with the GM line. Comparisons with historical or literary values have only limited scientific validity.

'Substantial equivalence' is a crude, non-scientific concept. It provides a loophole for the GM biotechnology companies not to carry out nutritional and toxicological animal tests to establish whether the biological effect of the GM crop-based foodstuff is substantially equivalent to that of its non-GM counterpart. It therefore allows them to claim that there is no need for biological testing because the GM crops are similar to their conventional counterpart, while on the other hand, because they contain novel genes from other organism(s), they are patentable. However, unintentional and unpredictable changes can occur in plants because of the incorporation and positioning of the vector in the plant genome. It cannot therefore be known which of the hundreds of components of the GM crop may carry toxic or allergenic properties. As most of these are unknown, by definition, they cannot be included in analytical comparisons. Determination of the amounts of protein, carbohydrates, fats and other nutrients can only be a starting point. The consumption of minor and unexpected constituents of potentially high biological activity may have considerable and disproportionately large effects on the digestive tract. Their presence, therefore, can only be revealed from animal studies, and this makes it imperative that these are performed with a flawless design and experimentation.

The FLAVR SAVR™ tomato was produced by 'antisense' GM technology. As part of its safety evaluation, it was subjected to compositional analysis for total protein, vitamins and minerals to establish whether any unexpected changes in gross fruit composition had arisen as a result of the integration of the FLAVR SAVR™ and *kan*^{r} genes into the tomato genome. It was claimed that no significant changes were found and that the contents of potentially toxic glycoalkaloids, particularly tomatine, and to a lesser extent solanine and chaconine, were also similar (Redenbaugh *et al*., 1992) and therefore this GM tomato was substantially equivalent to other non-GM tomato lines. However, to supplement these, several feeding studies were also performed by commercial laboratories at the request of the FDA.

Acute toxicity

First, range-finding, limit acute oral toxicity tests of the processed tomatoes in rats were carried out by the IIT Research Institute of the Life Sciences Department (Chicago, Illinois, USA). A single dose of the homogenates prepared from about 80 g of various GM and control tomatoes, respectively, was administered (15 ml kg^{-1}) by gavage to groups of Harlan Sprague–Dawley rats (five male or female rats per group) fed *ad libitum* on rat chow for 14 days to establish whether the GM tomatoes were toxic or not. As claimed, no test substance-related mortalities occurred and increases in mean body weights were not

significantly different between GM and control groups. However, as the range of the rats' starting weights was unacceptably wide (female rats weighed 131–186 g (± 18%) and male rats 159–254 g (± 23%)), in such a short (14 day) study with five rats per group, it would have been difficult for significant differences to develop. For comparison, only a few per cent variation in starting weights is permitted in papers published in high-quality nutritional journals. Thus, the poor design of this feeding study largely invalidated the conclusions that GM tomatoes were not toxic. To supplement these, three more rat feedings studies of similar design were carried out by International Research and Development Corporation (Mattawan, Michigan, USA).

Twenty-eight-day toxicology/histology study

Of the three studies, the most complete set of data is available for the second. In this, four groups of rats (20 males and 20 females per group) fed standard rat chow for 28 days were gavaged twice daily with homogenized tomatoes (15 ml kg^{-1}). Two groups were given GM tomatoes, CR3–613 or CR3–623 (CR3–623 is the commercial FLAVR SAVR™ tomato). There were two control groups, one of which was gavaged with the parent CR3 tomato homogenates and a second control group in which the rats were gavaged with water even though the relevance of this group is somewhat questionable. At the request of Calgene, an expert panel was retained (ENVIRON Corp., Arlington, Virginia, USA) to evaluate the data. They concluded that gavaging rats with GM tomato purée resulted in no significant changes in body weight, food consumption and clinical chemistry or haematology parameters in comparison with control tomatoes. However, there was a possible treatment-related increase in glandular stomach erosion/necrosis in four out of 20 female rats but none in the controls or in male rats at the end of the 28-day feeding period. The number of four female rats was increased to seven when the histology slides were re-scored by PATHCO, an independent pathology working group. This prompted a repeat study in which the dose of the tomato purée was increased by twofold. Unfortunately, in this study, some of the CR3 control and CR3–623 GM tomato lines were grown at different locations and harvested at different times from those in the second experiment. However, this was not regarded as important by the expert panel even though, when the same tomatoes were used as in the second experiment, the results appeared to show similar tendencies; two out of the 15 females developed stomach glandular erosions with the GM tomatoes, while none were found in the control females. However, in a not clearly understandable way, the ENVIRON panel concluded that the lesion of glandular erosion was not related to the administration of GM tomatoes. According to them, such lesions occur spontaneously in animals that are stressed or given mucolytic agents, when food is restricted or when animals are restrained in cages, even though these parameters have not been investigated systematically. Moreover, none of these circumstances applied, since tomatoes contain no mucolytic agents, food was provided *ad libitum* and the rats were not restrained. It was also suggested that, because the lesions were possibly of short duration, they were incidental, not related to the test material and would have healed spontaneously. Unfortunately, none of these assumptions was confirmed by further experimentation as no samples other than those at the end of the 28-day experiment were taken to probe into the timing and reversibility of the incidence of the stomach lesions. Clearly, the results of these three studies should have prompted more experimentation to investigate in more detail the effect of GM tomatoes on stomach histology and, what is even more important, these studies should have been extended to include the possible effects of GM tomatoes on both the small and large intestines.

The red or dark red pin-point lesions present in the stomach of female rats which were described as necrosis would be termed 'erosion' in human pathology, which may have sequelae, such as life-endangering

haemorrhage. Erosions cannot be termed 'mild', as unpredictable haemorrhage can occur in the elderly human, particularly on low dosage aspirin to prevent thrombotic events, and synergy with transgenic tomatoes may occur. The assumption that the lesions are related to stress does not explain the low incidence in other groups, particularly in the second study. The relevance and significance of gastric erosions in the human may be a matter of life and death in the older age groups. It has been implied that pathologists in general might not report such a lesion but, in the present era of vexatious litigation, mention would have been made in any human pathologist's report to avoid an accusation of negligence. This may not be required in veterinary pathology but these rat studies were done with humans in mind and therefore the pathology findings must be put in this human context. It is probably true to suggest that these lesions are of short duration, but the serious nature of erosive lesions should not be trivialized. This is the more serious because seven out of 40 rats eating GM tomatoes died within 2 weeks. The nature of these deaths was not specified and the evidence that they were not related to the ingestion of transgenic tomatoes was inconclusive.

In a further development, the Scientific Committee on Food of the European Commission Directorate C (2000) concurred with the conclusion reached in the US Food and Drug Administration (1994) memorandum. In their opinion, although the results showed an unexplained disparity, they were not supportive of a substance-related effect of the FLAVR SAVR™ tomato. However, it is likely that the EU Committee may not have seen all the primary data and their opinion would therefore have been based on incomplete evidence. It is also regrettable that, by ascribing the gastric erosions in rats to 'an artefact of gavage studies', the EU Committee has in fact labelled the scientists carrying out the work as incompetent. As these erosions were found at the end of a 28-day study during which 160 rats were gavaged twice daily with tomatoes, it is unlikely that even poorly trained workers would not have become more competent, so as to avoid causing such an anomaly.

Effects on body weight, food intake and organ weights

The conclusion of the ENVIRON panel that feeding rats on GM tomatoes (CR3–623) for 28 days had no effect on weight gain, feed intake and organ weights could not be justified because the starting weights of the rats were so widely different – a range of 130–258 g (± 33%) for males and 114–175 g (± 21%) for females – that finding significant differences in weight gain, feed intake and organ weights was not likely. Indeed, weight gains varied between wide limits (102–230 g for males and 46–127 g for females) in 28 days. Even under these conditions, although the average starting weight of the male rats gavaged with CR3–623 GM tomatoes was the highest (148.1 g), their final weight (316.5 g) was the lowest. Accordingly, the rats gavaged with GM tomatoes grew the least of the four groups of rats. The feed intake of the different groups also varied between wide limits; 133–203 g for males and 102–153 g for females. Not surprisingly, the feed conversion efficiency (weight gain/total feed intake) of female rats on GM tomatoes (0.152) was significantly ($P < 0.05$) less than that (0.167) obtained for female rats on control non-GM CR3 tomatoes.

The large range of starting weight differences also excluded the possibility of finding significant differences in the organ weights of the four groups of rats. The standard deviations of mean values were very large, in some instances more than 20%. It is the more remarkable that, even under these conditions, some differences in organ weights were found, including the testes for males and the thyroid/parathyroid for females. Finding no significant differences in biochemical, haematology and ophthalmology parameters between GM and non-GM tomatoes was not unexpected either, because of the large initial body weight differences.

Overall, it is regrettable that these rat toxicological feeding studies were poorly

designed, as a great deal of effort, work and money must have been spent on them and so much rested on the outcome. The FDA's conclusion that FLAVR SAVR™ presented no more dangers to consumers than ordinary tomatoes does not therefore appear to rest on good science and evidence which could stand up to critical examination. Rather tellingly, the results of these studies have never been published in peer-reviewed journals. The study as described not only raises questions about the design, methods and conclusions for this study but also whether they could have any general validity for other GM foods. In this light, it is the more surprising that after these studies the FDA has required no nutritional/toxicological testing of other GM foods.

Aventis's Chardon LL herbicide-resistant GM maize

Due to the UK government's attempt to place Chardon LL seed on the National List, a part of the supporting evidence submitted by Aventis contained data on the composition of two lines of seed to establish their substantial equivalence to the conventional parent maize line. The evidence also included the results of a 14-day rat feeding study. All this is to be found in a file deposited by the Ministry of Agriculture, Fisheries and Food (MAFF) with the British Library (British Library File, 1997).

Compositional analysis

In the absence of specifying the origin and conditions of cultivation of the different GM and non-GM samples, strict scientific comparisons could not be made between them. However, even under these conditions, the composition of T14 and T25 GM maize expressing phosphinothricin acetyltransferase enzyme (PAT-PROTEIN) showed many statistically significant differences in fat and carbohydrate contents in comparison with non-GM grain samples, and fat, protein and fibre between silage samples from GM and non-GM maize. Thus, the conclusion that GM maize is not 'materially different' from current commercial varieties cannot be regarded as valid.

Repeated dose oral toxicity (14-day feeding) study in rats

The rationale for this study was to assess the cumulative toxicity of PAT-PROTEIN given to rats in their diet for 14 days and to provide a rational basis for toxicological risk assessment in man. Although testing of the PAT-PROTEIN can be commended, this study was no substitute for the nutritional testing of the entire GM plant, seeds, vegetative parts and silage in all target animal species. Without these, the potentially harmful, unintended and unpredictable effects of the gene transfer, other components of the vector and gene insertion (positioning effect) cannot be established or excluded.

Unfortunately, as the design of the experiment was faulty, it is difficult to draw valid conclusions from a feeding study, carried out with five rats per group, in which the starting weight of the rats varied by more than ±20% (53–82 g for males and 50–74 for females) rather than the usual ±2%. For any differences to reach significance, they needed to exceed ± 20%, and to achieve this in a 14-day study would have required catastrophic experimental conditions. The five rats per group were not housed singly and therefore their individual feed intakes could not be monitored even though the huge differences in the starting weights should have led to major differences in the feed intakes of the individual rats. Moreover, the group feed intakes were not measured continuously. There were four groups of rats (five male/female rats per group) in the experiment. However, rats in group 1 were fed a different diet (full rat chow) from the other three groups and therefore group 1 was not appropriate for (statistical) comparisons. The diet of the second group contained 5 g kg^{-1} and the third group had 50 g kg^{-1} PAT-PROTEIN mixed in with 45 and 0 g kg^{-1}, respectively, of commercial (SOJAMIN, KLIBA Muhlen AG) low soybean protein diet (11% raw protein). The diet of the fourth group contained 50 g kg^{-1} SOJAMIN but no PAT-PROTEIN. Thus, for statistical analysis, the second and third groups ought to

have been compared with rats in the fourth group. Curiously, although the main target organ of the PAT-PROTEIN fed to rats was the digestive tract (and pancreas), the weights of these were not measured. This is a major experimental design fault.

The starting weight and the feed intake of the third group (high PAT-PROTEIN) were the highest, but they ended up with the lowest final body weight. This indicated an elevated metabolic activity, probably induced by the PAT-PROTEIN. Our analysis of variance (ANOVA) shows that the weight gain for both male and female rats on the high PAT-PROTEIN diet (group 3: 65.2 and 43.6 g for males and females, respectively) was significantly ($P < 0.05$) less than that of either the fourth group (control: 72.8 and 48.8 g for males and females, respectively) or group 2 (low PAT-PROTEIN diet: 73.4 and 44.4 g for males and females, respectively). As PAT-PROTEIN reduced feed conversion efficiency, it is potentially harmful. The conclusion that 'there were no differences which could be attributed to treatment with the test article' was therefore not valid. Similarly, that 'there were no changes on … clinical biochemistry and urine analysis after 14 days' is not valid either as the authors' own results described differences between the groups in glucose, cholesterol, triglyceride and phospholipid levels, indicating an increased metabolic functional load in the rats. It is unexplained why these differences were dismissed by the authors as incidental and unrelated to the treatment. Our ANOVA analysis revealed that the urine output in rats on the high PAT-PROTEIN diet was significantly ($P < 0.05$) reduced, indicating treatment-related effects (urine output of 5.4 and 4.4 ml for males and females in group 3 vs. 7.1 and 6.5 ml for males and females, respectively, in control group 4).

The large differences in the starting weight of the rats probably prevented finding significant differences in organ weights. However, even under these conditions, rats fed the high level PAT-PROTEIN diet (third group) had the lowest liver, thymus and spleen weights of all groups (even though the differences with controls were not significant). This is of particular importance because the macroscopic findings indicated thymus foci in 20–40% of the animals fed diets containing the PAT-PROTEIN.

In conclusion, the design and execution of this feeding study were poor and, contrary to the authors' conclusions, the results indicated treatment-related effects induced by PAT-PROTEIN (of unspecified origin). The results therefore could not be taken as evidence that the transfer of its gene into maize represented no risk for the rat and, by inference, for humans, particularly as no gut histology studies have been completed so far. Finally, a recent publication (Chiter *et al*., 2000) showed that DNA survived in intact form or slightly fragmented unless the GM maize was heat processed extensively. Therefore, the possibility exists that with underprocessed maize products humans and animals might be exposed to the DNA used in the genetic engineering.

Compositional Studies Published in Peer-reviewed Journals

Herbicide-resistant soybean

Befitting its importance in both human and animal nutrition, a great deal of attention has been given to the compositional analysis of herbicide-resistant and other GM soybeans. Several publications appeared in nutritional and other journals demonstrating the compositional 'substantial equivalence' of GM and non-GM soya. Thus, it was claimed that the macronutrient composition of glyphosate-tolerant soybean (GTS) seeds resulting from the transformation of conventional soybean with a gene encoding 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium*, to make the soya herbicide resistant, was equivalent to that of conventional soybeans. This applied equally to GTS unsprayed with glyphosate (Padgette *et al*., 1996) or sprayed with this herbicide (Taylor *et al*., 1999). It was claimed that the results of proximate chemical analyses of the contents of crude protein, oil, ash, fibre, carbohydrates and amino acids of solvent-extracted and toasted or untoasted soybean meals of unsprayed GTS and control soybean had shown that all these lines were

substantially equivalent (Padgette *et al*., 1996). Similar findings were described for sprayed GTS (Taylor *et al*., 1999). Although this appeared to be true for most macronutrients, several significant differences between GM and control lines, such as in ash, fat and carbohydrate contents, were also found (Padgette *et al*., 1996). However, these were not regarded as having biological significance.

A closer inspection of the data in the papers, however, revealed that the statistical comparison of the macronutrients of GM and non-GM lines was not scientifically valid. Instead of comparing their amounts in a sufficiently large number of samples of each individual GTS with its appropriate individual parent line grown side by side at the same location and harvested at the same time to establish whether they were 'substantially equivalent', what the authors compared was a large number of different samples from different locations and harvest times. As growth conditions have a major influence on seed composition, the range of the amounts of constituents in the different samples, regardless of whether they were GM or non-GM, was so great (±10% or more) that the chances of finding statistically significant differences were unreal. It is possible that from a practical point of view the variation in protein concentration of samples of the three lines of between 36.8 and 45% would fall into the normal range of agronomic variability of soybeans and therefore may not be of major concern for agronomists. However, this comparison is not strict enough to establish whether the genetic modification introduced any unintended compositional changes. What is remarkable is that, even with this approach, many significant changes in macronutrient levels were found. Thus, the claim of 'substantial equivalence' of GTS lines with non-GM soybean is not supported by rigorous scientific evidence.

The potential importance in human health of natural isoflavones, such as genistein, daidzein and coumestrol present in soybeans, is generally recognized. It was, therefore, of considerable interest whether any changes occurred in these components as a result of genetic modification. Here the published evidence is controversial. Thus, while in some studies no meaningful differences were reported (Padgette *et al*., 1996; Taylor *et al*., 1999), an independent study claimed that GM soya samples had consistently contained significantly fewer isoflavones than the parent cultivars (Lappe *et al*., 1999). In one respect, all authors agreed, i.e. that the isoflavone content of soybean seeds showed considerable variability between sites and was dependent on agronomic conditions. However, Lappe *et al*. (1999) went further and claimed that, while the variability of the GM samples was indeed considerable, conventional soybeans showed less variation in isoflavone content. As the isoflavone content of soybeans might affect human health, there needs to be more awareness of potential health problems due to this variability. While the precise details of the changes in isoflavone content on genetic modification will have to be established in the future, to ensure clinical consistency, the origin and the actual phyto-oestrogen levels in soybean may need to be standardized.

In the study by Padgette *et al*. (1996), no significant differences were found in the levels of antinutrients, such as trypsin inhibitors, lectin and oligosaccharide flatulence factors, between solvent-extracted, toasted or untoasted GM and non-GM soybean seeds. However, the comparisons were made by the same method as for macronutrients and therefore the large range of natural variability excluded the possibility of finding significant differences. Interestingly, in single soybean meal samples of each of the two GTS and parent lines, the trypsin inhibitor (also a major allergen in soybean) content was substantially higher, by almost 30%, in one of the two GTS lines, with a smaller increase in the other. No trypsin inhibitor analyses were performed on the protein isolate or protein concentrate samples originating from the meal samples. Although there were other compositional differences in these processed soybean products, it is difficult to decide from single determinations whether these were significantly different or not.

In conclusion, there is insufficient evidence to date to decide whether the composition of GM and conventional soybeans is equivalent or not. In fact, some data, particularly those for phyto-oestrogens, were

significantly different. Furthermore, because not strictly comparable compositional data were used, the case for equivalence was not properly established. There is therefore an obvious need for further more critical studies.

GM potatoes

Brief references to GM potatoes, particularly those expressing *Bacillus thuringiensis* (Bt) toxin, can be found in non-peer-reviewed book chapters or other articles. In most instances, these contain no data and are therefore of little scientific value. There are two exceptions, one of which is an article on the safety assessment of GM potatoes expressing the soybean glycinin gene (Hashimoto *et al*., 1999a). However, it is not quite clear what the authors wanted to achieve because, at the expression level of glycinin in potatoes of between 12 and 31 mg g^{-1} total soluble protein, no significant improvements in the protein content or amino acid profile could have been expected. Indeed, the results in the paper demonstrated that the total protein content of the GM potatoes appeared to be significantly less than that of the control line and that no improvement in the essential amino acid profile was achieved either. There appeared to be substantial differences in some vitamins between GM and control lines, and the amounts of both solanine and chaconine increased in the GM lines. It is, therefore, not quite clear why it was claimed by the authors that their GM lines were equivalent to the parent line and could be utilized as safely. The other more recent study is a conventional compositional analysis of some macro- and micronutrients of tubers from insect- and virus-resistant potato plants (Rogan *et al*., 2000) performed by methods which currently are accepted by most novel food regulatory bodies. Although these showed some significant differences in a number of tuber constituents, in the absence of toxicological/nutritional animal studies it is difficult to ascertain whether these differences could have any biological effects on humans/ animals, particularly as these conventional analyses could not have revealed the development of any unknown possible toxic/antinutritive components. Additionally, known antinutrients, such as lectins or enzyme inhibitors, were not included in the analysis.

GM rice

GM rice lines expressing the soybean glycinin gene have been developed (Momma *et al*., 1999) by a method similar to that used for GM potatoes. The glycinin expression level was between 40 and 50 mg glycinin g^{-1} total rice protein. The GM rice was claimed to contain 20% more protein, but its moisture content was less than that of the parent line. However, from the paper, it is not quite clear whether the increased protein content was due to the decreased moisture content of the seeds because it was not specified whether the values were expressed for air-dried or fully dried seeds. Thus, most of the arguments in the discussion of whether the higher protein level was due to the positioning effect of gene insertion or metabolic interference will have to await clarification by further work.

GM cotton

Several lines of GM cotton plants have been developed using the gene encoding an insecticidal protein from *B. thuringiensis* subsp. *kurstaki*. These had increased protection against the major lepidopteran insect pests of cotton. As cottonseed is an important source of oil for human consumption, and cottonseed and processed cottonseed meal for animal feed, extensive analytical work has been done to establish whether the GM lines were 'substantially equivalent' to conventional lines (Berberich *et al*., 1996). The levels of protein, fat, carbohydrate, moisture, ash, amino acids and fatty acids in the insect-protected lines were claimed to be comparable with those found in commercial varieties. Moreover, the levels of antinutrients such as gossypol, cyclopropenoid fatty acids and aflatoxin were similar to or less than those in conventional seeds. Thus, the GM varieties

were suggested to be equivalent to conventional seeds and just as nutritious. However, the statistics used by the authors were identical to those used with glyphosate-resistant soya and therefore could be similarly criticized. Although the content of known constituents fell in between the wide range of values of commercial conventional lines, this did not mean that they were compositionally equivalent, particularly as environmental stress could have major and unpredictable effects on antinutrient and toxin levels (Novak and Haslberger, 2000). Thus, without animal experimentation, this approach could not reveal whether any new and unknown toxins/allergens had been created or not.

GM maize

A glyphosate-tolerant (Roundup Ready) maize line GA21 has recently been developed. It was claimed (Sidhu *et al*., 2000) that, except for a few minor differences, which the authors think are unlikely to be of biological significance, the results of compositional analyses of proximate, fibre, amino acid, fatty acid and mineral contents of the grain, and proximate, fibre and mineral contents of forage collected from 16 field sites over two growing seasons showed that control and GM lines were comparable. The comparison was carried out by a method similar to that described for GTS soya (Padgette *et al*., 1996) and this may therefore not be scientifically rigorous enough for the establishment of substantial equivalence.

Nutritional/Toxicological Studies Published in Peer-reviewed Journals

Herbicide-resistant soybean

As part of a safety assessment of GTS, the feeding value, wholesomeness (Hammond *et al*., 1996) and possible toxicity (Harrison *et al*., 1996) of two major GM lines of GTS were compared with those of the parent line. Processed GTS meal was included in the diets of rats, broiler chickens, catfish and dairy cows at the same concentrations as in commercial non-GM soybean rations. Rats and dairy cows were fed these diets for 4, broilers for 6 and catfish for 10 weeks. It was claimed that in rats, catfish and broilers the growth and feed conversion efficiency, in catfish the fillet composition, in broilers the breast muscle and fat pad weights, and in dairy cows milk production and composition, rumen fermentation and digestibilities were similar for both GTS and parental lines. According to the authors, these results confirmed that the GTS and parental lines had similar feeding values.

Rat studies

A critical evaluation of the rat study was hampered by the lack of adequate primary individual data in the paper. Thus, there was no full description of the rat diet. It appears that the total protein content of the diets was adjusted to 247 g kg^{-1} diet to be isonitrogenous with Purina Laboratory Rat Chow by the addition of 24.8 g of GTS and parent soybean meals, respectively (~10% protein), to a base diet. All comparisons were made with rats fed commercial Purina Chow. The protein concentration in these diets was, however, appreciably higher than the usual 10–16% crude protein and exceeded the protein requirements of the rat. This extra protein potentially could have masked any possible transgene product effects, particularly with the raw unprocessed soybean diets in which the GM meals were incorporated only at the level of 50 or 100 g kg^{-1} of the diet. Thus, these meals only replaced 8.5 and 17%, respectively, of the total protein of 247 g kg^{-1} diet. In other words, the GM soybean protein in these meals was diluted by other dietary proteins by 12- and 6-fold, respectively, producing another possible masking effect. The composition of the control Purina Chow diet in the ground raw soybean feeding study was not described. This is important because the identity of the raw control soybeans included in the Purina Chow control diet was not specified.

In the feeding study, four groups of rats (ten males and ten females in each group) singly housed were fed diets containing the

parental line or the GTS lines (40-3-2 or 61-67-1) for 28 days. No individual values (or their ranges) for feed intake or body weight were given. The bar diagrams of the combined body weight of rats at the end of each week of the 4-week experiment were rather uninformative. However, it was observed by the authors that the Purina Chow-fed male rats grew significantly better than the three experimental groups fed toasted soybeans (including the parental line). This was attributed to better commercial processing. However, the bar diagrams also indicated that the growth in the group fed with one of the GTS lines (61-67-1) was probably equal to that of the Purina Chow-fed control and, therefore, by inference, these rats also grew significantly better than the other two experimental lines (the GTS line 40-3-2 and the parental line). This again underlined the importance of giving individual data in papers, without which it is difficult to assess the results. Similarly, there were no individual data for organ weights, such as liver, kidneys and testes. However, it was claimed that the kidney weights of the raw GTS line-fed (and parental control?) male rats were significantly higher than those of the controls, while the testes of the parental line-fed rats were significantly enlarged. According to the authors, as these differences were neither dose related nor only shown by the parental line, they were not caused by genetic modification. Rather curiously, the weights of the stomach and intestines, the main target organ in any nutritional testing, were not recorded. Observations were not recorded on other organs, and no histology appears to have been done on these tissues either. The only tissue which was subjected to microscopy was the pancreas, but the description of the findings was qualitative. Only minimal to mild lesions were found and these were claimed to be common to all groups. However, under these conditions, this was not surprising because no pancreatic hypertrophy was found. This was probably due to the effect of the unusually high dietary protein concentration, which, as the authors pointed out, masked and/or diluted the biological effect of the trypsin inhibitors. This is of particular concern because the trypsin inhibitor content of GTS lines in unprocessed soybean was significantly higher than in the control line (Padgette *et al*., 1996).

It is regrettable that the design of this important rat feeding study had such unfortunate omissions. It is of particular concern that no histology was apparently carried out on gut tissue. Thus, more critical work is needed to decide whether the feeding value of GM and non-GM soybeans is equal or not.

Chicken study

The broiler chicken feeding study's experimental design closely followed that of commercial practice and therefore the results should only be indicative of the commercial feeding and production value of the various soybean lines. As the data were pooled from all birds fed on the same diet, it is not easy to see what, if anything, was the significance of the small differences found in the study, such as the slightly lower body weights, breast and fat pad weights obtained with the GTS lines (particularly with GTS 40-3-2) for the utilization of GM soybean. It would have been better to measure the nutritional performance of individual birds (or small groups) fed on different diets and then compare them after statistical analysis. In the absence of this, we have to rely on the authors' conclusion that the design of the experiment gave the upper limit of differences in weight gain of 3.5% and gain/feed ratio of 2% and that the GTS lines vs. parental line were within this limit. Thus, with this restriction, the feeding value of the GTS lines for broilers was practically equal to that of the parental soybean line.

Catfish experiment

Catfish are excellent and highly sensitive indicators for the feeding value of diets. It was obvious from the results that, similar to the findings with rats, one of the GTS lines, 61-67-1, was superior to the other lines (GTS 40-3-2 and the parental line) in most respects. Thus, fish on GTS line 61-67-1 ate more, had better weight gain and gain/feed ratio and weighed more at the end of the 10-week study than the others, even though the composition of the fillets from these fish was not significantly different. This significant

difference in performance must, therefore, indicate that genetic modification may not be as reproducible as it has been claimed and that the feeding value and metabolic effects of GM and parent lines are not always 'substantially equivalent'.

Study on lactating cows

Milk production and composition and performance data in the lactating cow study showed some significant differences between cows fed diets containing the different lines of soybean, indicating a lack of 'substantial equivalence'. In view of these differences, even though we may not at present know all their biological/nutritional consequences, it may be difficult to maintain the view that the feeding value of the GTS and parent lines is equal, and further work is needed to establish whether the GTS lines are safe or not for humans/animals.

Testing of E. coli *recombinant gene product*

Extensive studies have been carried out to ascertain the safety of the gene product, 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), which renders the soybeans glyphosate resistant (Harrison *et al*., 1996). Unfortunately, there are some flaws in these experiments, the most important of which is that in the acute gavage studies the authors did not use the enzyme isolated from GTS lines but instead that from *Escherichia coli*. Although they were at pains to show that the EPSPS enzyme samples from the two sources were similar in lack of glycosylation, molecular size, reaction with a polyclonal anti-EPSPS antibody and enzyme assays, these methods do not have sufficient power to show unequivocally whether they were identical. The authors themselves pointed out that post-translational modification of the completed polypeptide chains emerging from the ribosomes may be done differently in two such evolutionarily distinct life forms as higher plants and prokaryotic bacteria. Amidation, acetylation and proteolytic processing can have such major effects on the conformation of the protein as to make these gene products behave differently in the digestive system. Thus, the use of the *E. coli* recombinant protein for the acute mice gavage studies may invalidate the authors' conclusion that the gene product from soybean did not have any toxic effects. These studies must be re-done with the gene product isolated from the transgenic plant before the results could be accepted. In any case, in such gavage studies, young, rapidly growing animals must be used to show any distinct effect on growth. As all animal weights were unchanged in the experiment, the test system used could not have detected any effect unless the consequences of the gavaging had been disastrous. Feeding studies with the gene product in young rapidly growing rodents should be the preferred method for the demonstration of the deleterious effects.

The other flaw in the experimental design was the reliance on an *in vitro* simulated gastric/intestinal digestion assay, which was also carried out with the *E. coli* recombinant gene product. To obtain physiologically valid results, it would have been necessary to use the gene product isolated from GM soybean in an *in vivo* assay in the rat (or other suitable animals; see Rubio *et al*., 1994) or a full feeding trial. Thus, it has been shown before that the kidney bean (*Phaseolus vulgaris*) α-amylase inhibitor is fairly stable to proteolytic degradation in the rat gut (Pusztai *et al*., 1995, 1999), but, when its gene was expressed in peas (*Pisum sativum*), it was rapidly digested and inactivated in the rat stomach/small intestine *in vivo* (Pusztai *et al*., 1999). This may have contributed to the safety of GM peas for rats and, by inference, possibly for other monogastric mammals. Thus, *in vitro* digestion assays may have little relevance to the safety of GM food crops.

In a separate feeding study (Teshima *et al*., 2000), the possible harmful effects of toasted glyphosate-resistant GM soybean were investigated at 300 g kg^{-1} inclusion level in the diet of rats and mice. After feeding these animals for 15 weeks, no significant differences in nutritional performance, organ development, histopathology of the thymus, liver, spleen, mesenteric lymph nodes, Peyer's patches and small intestine, and the production of IgE and IgG humoral antibodies between GM and non-GM line diets were

found. However, as rats grew less than 30 g and mice not at all in 15 weeks, the conditions were so unphysiological that no valid conclusions could be drawn from these experiments.

GM maize

In a major commercial-scale broiler chicken feeding study with rations containing transgenic Event 176-derived Bt maize involving 1280 birds (Brake and Vlachos, 1998), it was claimed that no statistically significant differences in survival or bird weights between birds fed diets containing GM maize, Event 176, or an isogenic parent maize line were found. Indeed, birds fed GM maize rations appeared to have significantly better feed conversion ratios and an improved yield of breast muscle. However, the authors cautioned against the conclusion that this enhanced performance could be attributed to the Bt maize *per se*. It is possible that the results might have been due to slight differences in the overall composition of the diets. This is reasonable considering the length of this study and possible problems of consistent diet preparation on a commercial scale. Minor differences in composition such as the slightly lower protein content of the GM maize and fat contents of the diets magnified to the scale of this trial make the results more relevant to commercial than to academic scientific studies.

In a poultry feeding study, it was claimed that the GA21 Roundup Ready maize-based diets gave similar performance data in growth, feed efficiency and fat pad weights to diets containing the parental control line (Sidhu *et al.*, 2000). However, this and a similar study carried out in Germany with a maize line expressing PAT-PROTEIN (Flachowsky and Aulrich, 2001) were commercial production experiments and made little contribution to scientific safety assessment.

In a separate study, maize was genetically modified by the transfer of the gene of egg white avidin to make the seed resistant to storage insect pests (Kramer *et al.*, 2000). It was also claimed that this GM maize was safe for mice as apparently, when, instead of a balanced diet, they were fed solely on this crop, the mice suffered no ill effects. However, the mice used in the experiment were adults which did not grow at all, and therefore the conclusion that the GM maize was safe is, at best, premature.

GM peas

Diets containing transgenic peas expressing the transgene for insecticidal bean α-amylase inhibitor (~3 g kg^{-1} peas) at two different inclusion levels in the diet, 300 or 650 g kg^{-1}, were subjected to nutritional evaluation with rats in a 10-day feeding trial (Pusztai *et al.*, 1999). The nutritional performance of rats fed GM pea diets was compared with those obtained with rats pair-fed iso-proteinic and iso-energetic diets containing parent-line peas and also lactalbumin diets spiked with isolated bean and pea α-amylase inhibitors, respectively. At 300 g kg^{-1}, but not at 650 g kg^{-1} inclusion level, the nutritional value of diets containing transgenic or parent peas was not significantly different. Even at 650 g kg^{-1}, the difference was small, mainly because the transgenically expressed pea recombinant α-amylase inhibitor was quickly (in < 10 min) degraded in the rat digestive tract and therefore its antinutritive effect was abolished. In contrast, spiking the parental line pea diet with the stable bean α-amylase inhibitor reduced its nutritional value (Pusztai *et al.*, 1995, 1999).

In this study, unfortunately, no gut histology was done or lymphocyte responsiveness measured, and therefore one had to rely on the evaluation of nutritional parameters that are inherently less sensitive in order to find possible differences in metabolic responses between GM and conventional food components. Although there were significant differences in the development of some organs, mainly the caecum and pancreas, most organ weights were remarkably similar. At the end of the study, cautious optimism was expressed that GM peas could be used in the diets of farm animals, particularly at the low/moderate levels recommended in

commercial practice and if the progress of the animals was monitored carefully. However, this relatively short feeding study with modest objectives cannot at this stage be taken as proof of the safety of GM peas for human consumption. There is a need to carry out further and more specific risk assessment testing procedures, which must be designed and developed with human consumers in mind. It also has to be kept in mind that only one particular line of GM peas was tested in which the endogenous antinutrient levels were selected to be similar to those of the parent peas. In some other GM lines, however, lectin levels could vary, up or down, by a factor of four. Moreover, in some field pea cultivars, such as Laura, the concentration of trypsin inhibitor increased by about 24% and the chymotrypsin by 100%, while the haemagglutinating activity decreased by a factor of four in the GM line compared with its parent (A. Pusztai, unpublished). This strengthens the argument that, in the safety assessment of GM crops, many lines should be included and that, from the results of a single GM line, no blanket approval should be given to other lines developed, even if in the transformation the same vector was used and carried out at the same time.

GM potatoes

There have been four independent studies on different GM potatoes.

Glycinin-expressing potatoes

The safety of transgenic potatoes expressing the soybean glycinin gene was evaluated in a short (4-week) rat feeding study (Hashimoto *et al*., 1999b). With an interesting experimental design, control rats and the experimental groups were fed the same control commercial diet. However, the rats were also daily force-fed by gavage with 2 g of respective potato lines kg^{-1} body weight. The potatoes used were a parental control line and two transformed lines, one with the glycinin gene and another one with a designed glycinin gene (coding for four additional methionines in the gene product), respectively. However, there were a number of problems with this study. Thus, although no difference in growth, feed intake, blood cell count, blood composition and internal organ weights between the groups was found, the uncertainty as to whether the animals were fed with raw or boiled/baked potatoes leaves a question mark over the interpretation of the results.

Bt toxin potatoes

An interesting, mainly histology, study was carried out on the ileum of mice fed with potatoes transformed with a *B. thuringiensis* var. *kurstaki Cry*I toxin gene. As a control, the effect of the toxin itself was also investigated (Fares and El-Sayed, 1998). It was shown that both the delta-endotoxin and, to a lesser extent, the Bt potato caused villus epithelial cell hypertrophy and multinucleation, disrupted microvilli caused mitochondrial degeneration, increased numbers of lysosomes and autophagic vacuoles, and increased activation of crypt Paneth cells. As a result, it was recommended that 'thorough tests of these new types of genetically engineered crops must be made to avoid the risks before marketing'. Unfortunately, some flaws in the experimental design detract from the strength of the conclusions. The most important of these was that, apart from indicating that the gene used in the transformation was the *Cry*I gene from *B. thuringiensis* var. *kurstaki*, there was no description of the Bt potatoes. The gene expression level in the GM potato was not given and it was not clear whether the potatoes in the diet were cooked or raw. Moreover, the amount of the Bt toxin used for supplementing the potatoes within the control potato diet was not specified either. This made it impossible to make a quantitative comparison of the effects on the ileum of the Bt potato with those of the spiked control potato diets. The assumption that the ileum is the most important absorptive part of the rodent small intestine could also be argued against, because 90% of all nutrient absorption in fact occurs in the jejunum. As this was an electron microscopy study, the fixation of the ileal samples was

not done on well-oriented sections but on chopped up fine tissue pieces, and important detail of villus organization was therefore lost. Finally, the delta-endotoxin-induced hyperplastic changes on ileal villi should have been demonstrated by measuring cell proliferation and mitotic rates in ileal (and jejunal) crypts rather than on the villi. However, despite these shortcomings, this study has established once and for all that, in contrast to general belief, exposure of the mouse gut (ileum) to the *CryI* gene product has caused profound hypertrophic and hyperplastic changes in cells of the gut absorptive epithelium and can lead to mucosal sensitization (Vazquez Padron *et al*., 1999, 2000b). This shows up the fallacy of drawing comforting conclusions from *in vitro* simulated gut proteolysis tests. Clearly, concerns about the possible biological consequences of exposure to GM food should be addressed under *in vivo* conditions because, even if an *E. coli* product breaks down *in vitro*, this does not necessarily mean that the same gene product expressed in the transgenic crop should also break down.

GNA GM potatoes

Some of the results of rat feeding studies with GM potatoes expressing the snowdrop (*Galanthus nivalis*) bulb lectin (GNA) gene were similar to the results of Fares and El-Sayed (1998). A part of this work concerning the effect of GNA GM potatoes on the histology of different compartments of the rat gut was published (Ewen and Pusztai, 1999a). Although this peer-reviewed scientific paper was criticized by some, most of the criticisms were unpublished personal opinions. Moreover, most of the published criticisms (e.g. Kuiper *et al*., 1999) were answered adequately (Ewen and Pusztai, 1999b). Some selected results of the nutritional/metabolic studies were, against the wishes of the authors, placed on the website of The Rowett Research Institute (www.rri.sari.ac.uk), where most of the work was done (Bucksburn, Aberdeen, UK). However, so as not to jeopardize their eventual proper publication, these results will only be mentioned briefly.

Young, rapidly growing rats (starting weight of 84 ± 1 g) were strictly pair-fed on iso-proteinic (60 g total protein kg^{-1} diet, most of which was from potatoes) and iso-caloric diets (in contrast to that described in Kuiper *et al*., 2001) supplemented with vitamins and minerals for 10 days. The test diets contained either raw or boiled GM potatoes. The control diets contained the same amount of parental-line potatoes (raw or boiled) alone or supplemented with GNA at the same concentration as expressed in the GM potatoes. A positive control group of rats was also included in the experiment, and these were fed a lactalbumin-based high quality control diet to check for any potential problems in rat behaviour and experimental conditions. As part of the nutritional/metabolic evaluation, samples of stomach, jejunum, ileum, caecum and colon were taken, fixed and stained with haematoxylin and eosin for full quantitative histological evaluation (Figs 16.1, 16.2 and 16.3) or reacted with GNA antibody and subsequently stained using a PAP (peroxidase–antiperoxidase) method to establish whether any GNA was bound to the epithelial surface (Fig. 16.4). By measuring the mucosal thickness of the stomach and the crypt length of the intestines (Ewen and Pusztai, 1999a), it was shown that proliferation in the gastric mucosa was in part caused by GNA, the gene product. However, the growth-promoting stimulus on the small intestine of diets containing GM potatoes leading to crypt enlargement and a part of the stomach enlargement was not a GNA effect. As shown before and confirmed here, there was a slight binding of GNA to the small intestinal epithelium (Fig. 16.4). However, GNA is not a mitotic lectin and therefore it did not induce hyperplastic growth in this tissue (Pusztai *et al*., 1990). Accordingly, the jejunal growth was probably due to some as yet unknown effects of other parts of the genetic construct used for the transformation or the genetic transformation itself. Hyperplasia was shown previously by measuring the increase in crypt length (Ewen and Pusztai, 1999a). However, similar results were obtained by measuring the increase in crypt cell numbers (Table 16.1) and crypt mitotic figures (not fully significant) in the jejunum of GM potato-fed rats (Table 16.2).

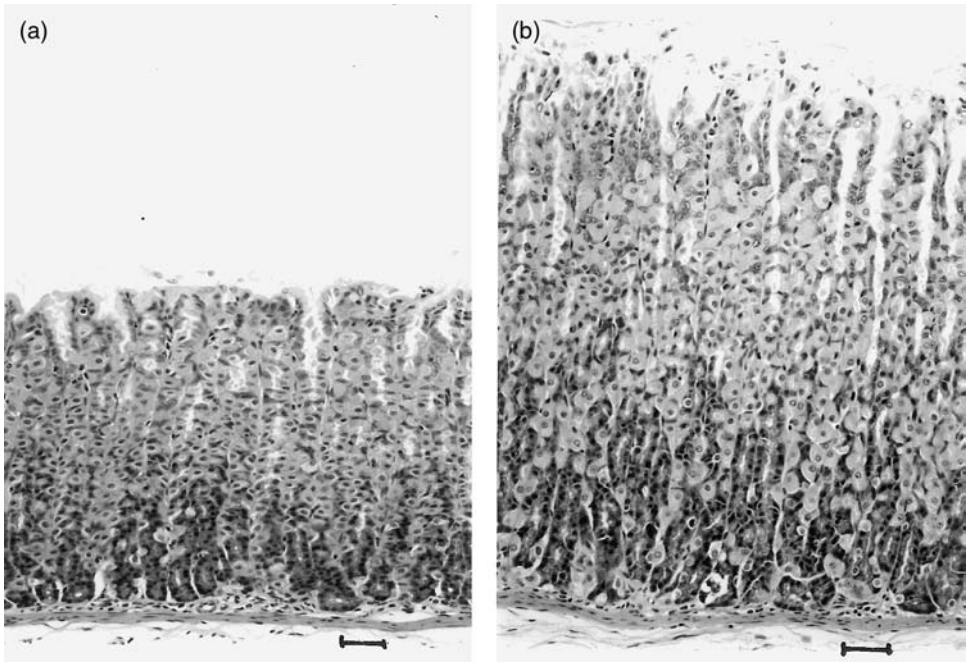

Fig. 16.1. Comparison of the stomach mucosa of rats fed with raw GM potato diet (b) shows marked thickening due to hypertrophy of mucosal cells in comparison with that of rats given the parental line (a) (bar = 100 μm).

The results suggested that it is possible that crypt hyperplasia and an increase in epithelial T lymphocyte infiltration observed with GM potatoes might also happen with other GM plants that had been developed using the same or similar genetic vectors and method of insertion. It is therefore imperative that the effects on the gut structure and metabolism of all GM crops should be investigated thoroughly as part of the regulatory process before their release into the human food chain.

Potatoes expressing cationic peptide chimeras

Desiree and Russet Burbank potatoes expressing N-terminally modified cecropin–melittin cationic peptide chimeras and control line potatoes fed to mice caused severe weight loss. The animals did not grow even after supplementing these potatoes with rodent laboratory chow. According to the authors (Osusky *et al*., 2000), mice fed with tubers from transgenic potatoes were as healthy and vital (*sic*) as those from the control group, and their faecal pellets were comparable. However, the severe weight loss seriously called into question the value of the results of this poorly designed feeding experiment.

GM tomatoes

Finally, an important study will have to be described even though it was not published in a peer-reviewed journal, but the ideas and experiments described had some influence on the development of GM regulation (Noteborn *et al*., 1995). Thus, a new laboratory GM tomato line was developed using the *B. thuringiensis* crystal protein CRYIA(b) gene but, instead of the cauliflower mosaic virus 35 S promoter (CaMV 35 S), which is used in practically all first-generation GM crops, a potentially safer plant promoter was used. Although with this the expression level of the Bt toxin was only about 1/20th of that found with CaMV 35 S, this might be improved upon in future. In contrast with most other studies with GM crops, there was a

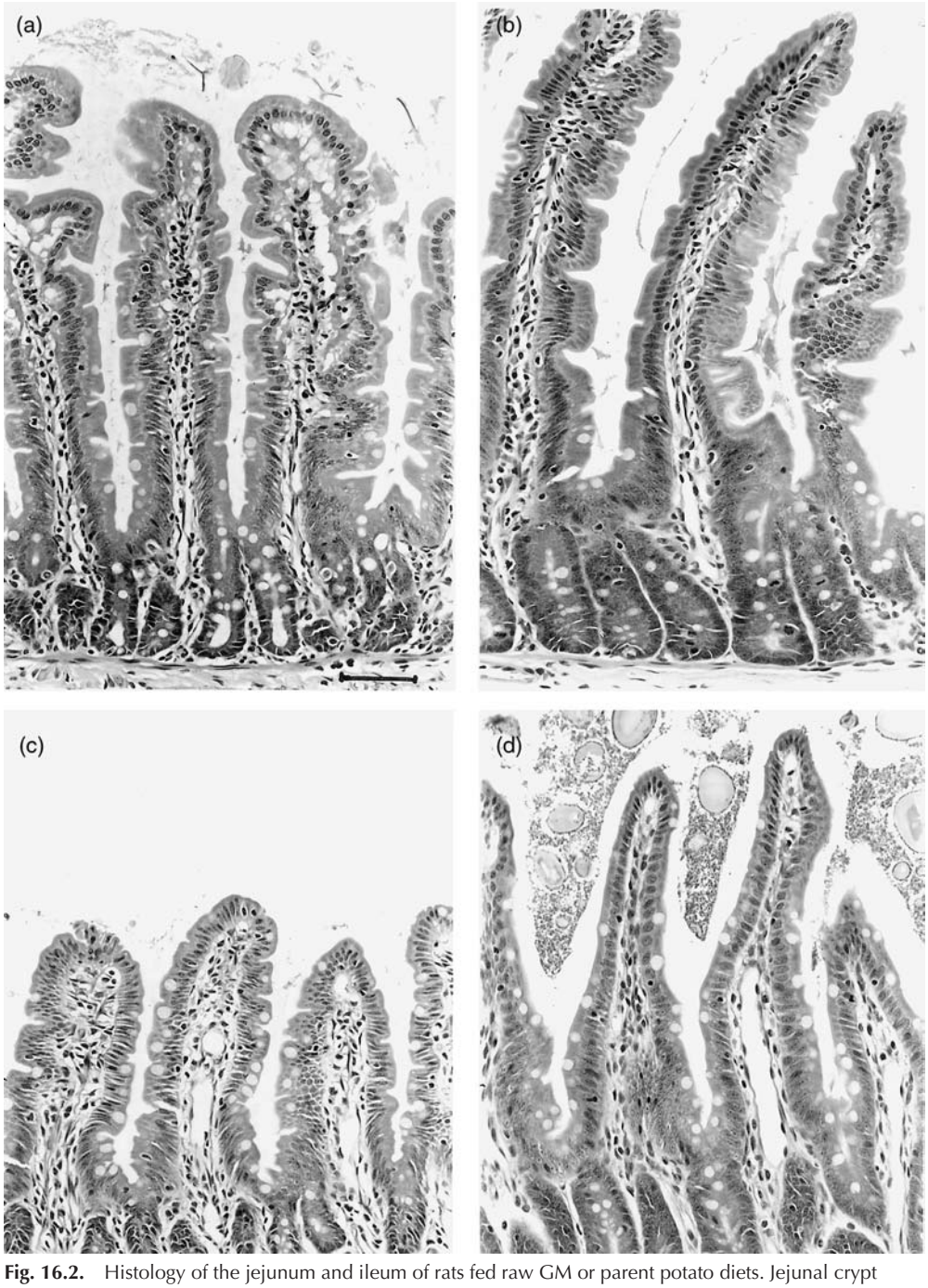

Fig. 16.2. Histology of the jejunum and ileum of rats fed raw GM or parent potato diets. Jejunal crypt length and cells exhibit marked enlargement after feeding rats GM potato diets for 10 days (b) in comparison with those of rats given parental line potato diets (a). The villus length is similar in both, but intraepithelial lymphocyte cell counts appear to be increased on the GM potato diet. In the ileum, both crypts and villi of rats on GM potato diets are elongated (d) in comparison with parent potato-fed rats (c) (bar = 100 μm).

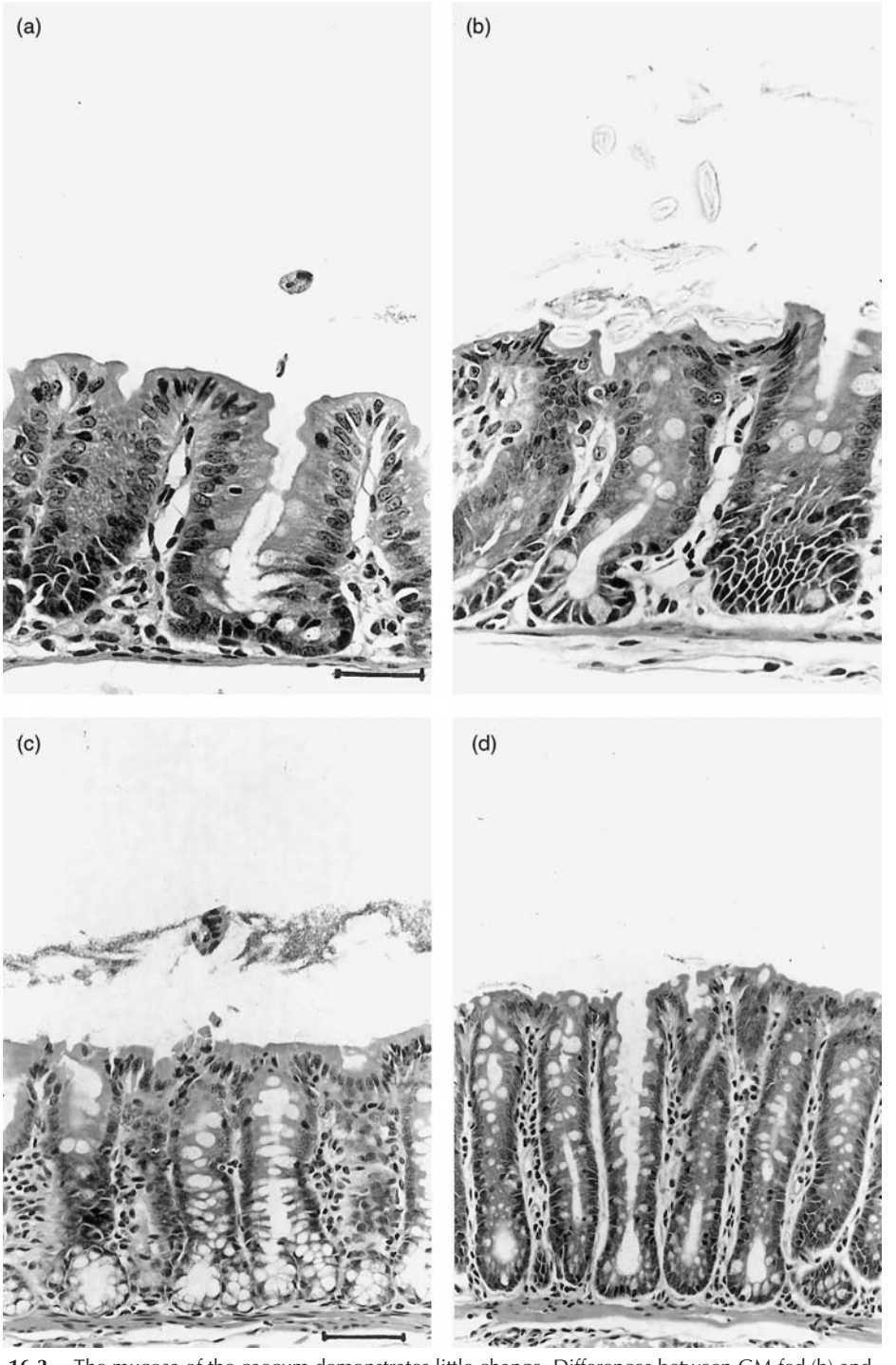

Fig. 16.3. The mucosa of the caecum demonstrates little change. Differences between GM-fed (b) and parent line potato-fed rats (a) are slight, while the colonic mucosa is moderately thickened in GM-fed rats (d) compared with that of rats given the parental line (c) (bar = 50 μm).

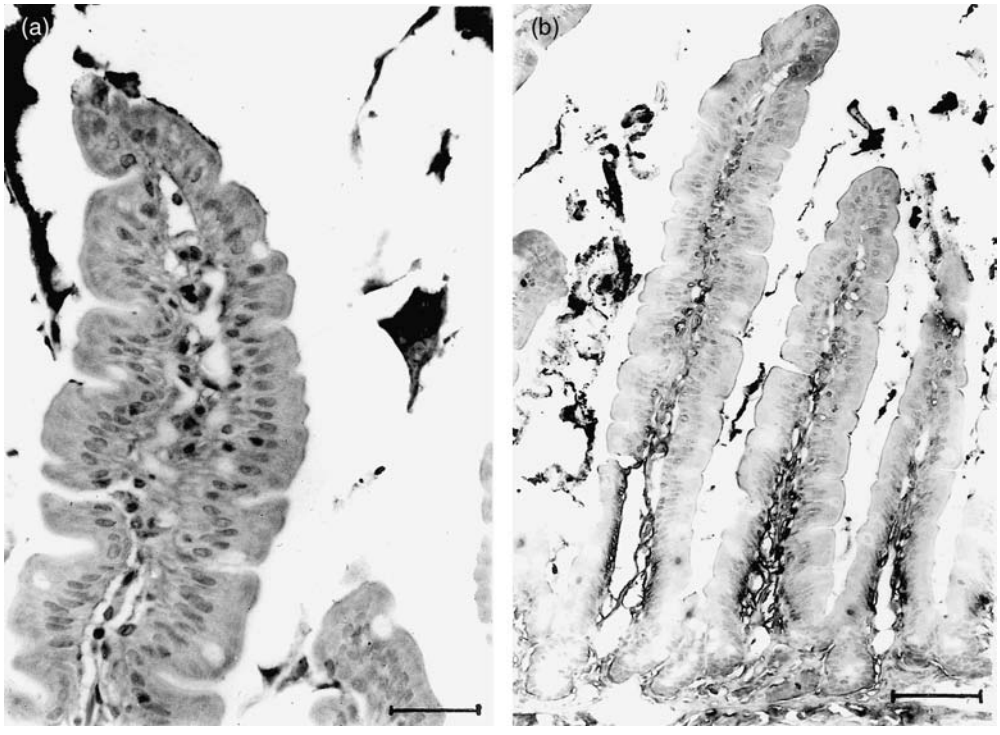

Fig. 16.4. Immunocytochemistry of the jejunum of rats fed raw GM potato diets for 10 days (a) shows moderate binding of GNA to villus tips (bar = 50 μm). Similar binding of GNA to jejunal villus tips is found in rats given parent potato diet supplemented with GNA in amounts equivalent to that expressed in the GM potato (b) (bar = 100 μm). Sections were first treated with anti-GNA rabbit antibody (diluted 1/100), followed by visualization with PAP. Note the strong antibody reactivity of feed particles in the sections.

Table 16.1. Number of crypt cells in the jejunum of rats fed various potato diets.[a]

Diet	Parent	Parent vs. parent + GNA (*P*)	Parent + GNA	Parent vs. transgenic (*P*)	Transgenic	Parent + GNA vs. transgenic (*P*)
Raw	15.9 (0.5)	0.037	17.0 (0.7)	0.000	20.3 (0.8)	0.006
Boiled	17.8 (1.1)	0.466	18.2 (0.2)	0.749	18.2 (1.2)	0.769
Raw vs. boiled (*P*)	0.006		0.003		0.003	

[a]The number of nuclei were counted sequentially on well-oriented haematoxylin and eosin paraffin sections (4 μm). Values represent means (SD) for six rats per treatment; ten crypts per rat were counted. Differences between treatments are significant when $P < 0.05$ (Student's *t* test). The effect of boiling ($P = 0.759$) is not significant, while that of GNA added or as transgene product ($P = 0.019$) and the effect of transformation ($P = 0.000$) are highly significant. The interactions between GNA and cooking ($P = 0.043$) and between transformation and cooking ($P = 0.018$) are also significant (multivariant analysis with Tukey's test).

commendable attempt to measure the binding of the gene product to the rat gut surface *in vivo* rather than using spurious arguments as to why the gene product should not bind. Although no *in vivo* binding was found, this should not detract from the significance of this initiative because, due to the lack of availability of sufficient quantities of Bt toxin

Table 16.2. Mitotic numbers per 100 crypts in the jejunum of rats fed potato diets.[a]

Diet	Parent	Parent + GNA	Transgenic
Raw	48	49	75
Boiled	57	56	57

[a]Mitotic cells were expressed per 100 crypts.

isolated from GM tomatoes, an *E. coli* recombinant and potentially less stable form of the gene product was used in the experiment, and its possible degradation in the gut may have accounted for the lack of binding. However, Bt toxin was shown by immunocytochemistry to bind to gut sections, including the caecum and colon, from humans and rhesus monkeys *in vitro*. Unfortunately, their short-term toxicity testing in mice (and rabbits) and the *in vitro* simulated proteolysis assays were also carried out with the *E. coli* recombinant gene product and therefore their conclusions of finding no toxic effects may not be valid. Commendably, the authors carried out a 91-day feeding study with rats using freeze-dried GM vs. parent line tomatoes, which were included at a 10% level in the diets, but no differences in food intake or body and organ weights were found. However, because the Bt toxin expression level in the tomatoes was low, the daily intake of the gene product(s) by the rats was also low. Moreover, as the daily input of tomato proteins was only about 5–6% of the total dietary protein intake of the rats, it was somewhat optimistic to expect any significant changes in these nutritional parameters. To have any reasonable chances to show up small differences in the nutritional value of GM vs. parent line crops, it would have been important to use as high a protein concentration as possible such as that in the 110-day GM potato feeding study carried out at The Rowett Institute, in which the GM protein in the diet was diluted only twofold by other dietary proteins, and this allowed the significant differences in the growth rates of rats fed on baked GM potato diets vs. parent potato diets to show up. In fact, to equalize the growth rates of the rats on the GM potatoes to that of the controls, the GM diet had to be supplemented with an extra 12 g lactalbumin kg^{-1} diet, and this extra protein gave a quantitative measure of the difference of the nutritional value between GM and non-GM potatoes. Even at these similar growth rates, the weights of some of the rats' vital organs, such as the gut and particularly the small intestine, the liver and kidneys, were still significantly different.

There were other omissions in the Bt tomato study, the most important of which was that no Bt toxin survival was measured in the gut lumen and no gut histology was done to see if there was any Bt toxin binding to or possible structural changes in the gut epithelium or whether lymphocyte infiltration occurred. This omission is particularly important because later studies showed that the similar Bt toxin Cry1Ac could bind to gut epithelial cells in mice (Vazquez Padron *et al*., 2000a,b) and induce mucosal antigenic sensitization (Vazquez Padron *et al*., 1999, 2000a,b). The allergenic potential of Bt tomatoes was not investigated either. However, despite some of its shortcomings, this study showed many novel and commendable features, which, after some improvements, may, hopefully, be incorporated into the general GM food testing procedures.

Allergenicity

One of the major health concerns with GM food is its potential to increase allergies in the human population through the food chain. The possibility of fatal anaphylaxis in sensitized individuals after their unwitting exposure to allergenic proteins in unlabelled GM foodstuffs is a real danger. When a gene is transferred from a source of known allergenic potential, the assessment of the allergenicity of the GM crop is relatively straightforward. This can be done using *in vitro* tests with sera from individuals sensitized to the allergen from the original source. Similarly, it is relatively easy to assess the effect of genetic engineering on endogenous allergens in crops with some evidence of allergenicity. With tests such as the radioallergosorbent test (RAST), RAST inhibition

and immunoblotting, the allergenic potential of the GM crop is easily measured. There are now several examples for these, such as the demonstration of the allergenicity of the Brazil nut 2S seed storage protein in transgenic soybean (Nordlee *et al*., 1996) or the codfish allergy in potatoes genetically engineered with cod protein genes to make the potatoes tolerate cold storage (Bindslev-Jensen and Poulsen, 1997). The claim that in glyphosate-tolerant soybean the introduction of the herbicide resistance gene does not affect the allergenicity of the soy endogenous allergens is also a good example (Burks and Fuchs, 1995). Having shown in a surveillance programme of farm workers before and after exposure to *B. thuringiensis* pesticide sprays that some developed skin sensitization and IgE antibodies to the Bt spore extract and that two of them had a positive skin-prick test, it may now be possible to test for the allergenicity of Bt toxins engineered into various crops (Bernstein *et al*., 1999). This is all the more important because the Cry1Ac toxin has now been shown to be a potent oral immunogen and adjuvant (Vazquez Padron *et al*., 1999, 2000a,b).

It is much more difficult to assess the allergenicity of GM foods when the gene is transferred from a plant whose allergenic potential is unknown. Moreover, it is also possible that, as a result of the gene transfer or vector insertion, a new allergen is developed or the expression level of a minor allergen is increased in the GM crop. The gene product can also have an allergenic adjuvant effect on a food component previously of low allergenic potential, or some component in the GM food may have an adjuvant effect on the allergenicity of the transgene product. Unfortunately, while there are good animal models for nutritional/toxicological testing, no satisfactory animal models have been developed so far for allergenicity testing (Helm and Burks, 2000). For the time being, only indirect methods are available for the assessment of the allergenic potential of GM foods derived from sources of unknown allergenicity. There are a number of recommended approaches to be followed. A useful preliminary step is to establish if there are any sequence homologies in the transgenic protein to any of the about 200 known allergens. If there are, *in vitro* tests for IgE reactivity need to be performed. It is thought that the peptide length in the transgenic protein which is optimally needed for binding B-cell epitopes requires the presence of at least eight contiguous identical or similar amino acids. However, the amino acids in the allergenic epitopes are rarely contiguous. Moreover, the absence of a positive reaction in *in vitro* testing does not guarantee that the transferred protein is not an allergen. In a decision-tree type of indirect approach, the next step is to consider the molecular size, glycosylation, stability, solubility and isoelectric point of the transgenic protein and compare them with those of known allergens (O'Neil *et al*., 1998). Unfortunately, in most studies to date, the all-important stability of the transgenic protein to gut proteolysis is established in an *in vitro* simulated gastric/intestinal system (Astwood *et al*., 1996; Metcalfe *et al*., 1996), and this is fundamentally flawed. The results, therefore, are at best misleading and at worst erroneous. Reliance on the concept that most allergens are abundant proteins is probably also misleading because, for example, Gad c1, the major allergen in codfish, is not a predominant protein (Bindslev-Jensen and Poulsen, 1997).

When the gene responsible for the allergenicity of a crop is known, its cloning and sequencing open the way for its reduction by antisense RNA strategy. Thus, in rice, the low molecular weight α-amylase/trypsin inhibitors are major allergens. A part of the genomic sequence encoding this protein in an antisense direction was constructed between the promoter of the rice allergen gene and its waxy terminator, and this was introduced into rice protoplasts. The allergenicity of the regenerated plants was significantly less than that of parental wild-type rice (Nakamura and Matsuda, 1996).

In conclusion, allergenicity testing appears to be one of the Achilles heels of GM food safety. It is clear that, if and when it is known that the protein gene is derived from a source with a history of allergenicity, there is a reasonable certainty that the GM crop will be allergenic. Unfortunately, the reverse is not true: the use of a gene from something that is not allergenic will not guarantee that the GM

crop will not possess allergenicity. In the absence of new and reliable methods for allergenicity testing, particularly the lack of good animal models, at present it is almost impossible to establish definitely whether a new GM crop is allergenic or not in advance of its release into the human/animal food/feed chain.

Conclusions

One has to agree with the opinion expressed in *Science* (Domingo, 2000) that there are many opinions but very few data on the potential health risks of GM foods, even though research to exclude such risks should have been carried out before the GM crops were introduced into the food chain. Our present database is therefore woefully inadequate. This is clearly seen from a closer scrutiny of the reference lists of recent reviews which contain only a handful of toxicological/nutritional and immune studies of GM food crops published in peer-reviewed science journals (Ruibal-Mendieta and Lints, 1998; Betz *et al*., 2000; Kuiper *et al*., 2001; Pusztai, 2001). Moreover, the scientific quality of even what is published is, in most instances, not up to the standards that ought to be expected. In this review, data published in peer-reviewed and some non-peer-reviewed journals have been examined in detail. However, as our future is claimed to be dependent on the success or failure of the promise of genetic modification delivering GM foods which will be wholesome, plentiful and, most importantly, safe for us all, the emphasis was on strict but fair criticism.

From the results, the conclusion seems inescapable that the present crude method of genetic modification has not delivered GM crops that are predictably safe and wholesome. The promise of a superior second generation of GM crops is still in the future. It is possible that some of the first generation of GM crops may superficially satisfy some commercial end points, such as their use in broiler chicken production. However, we need to consider that these GM feed ration-fed animals eventually will be consumed by humans, and there is absolutely nothing known about the potential hazards (if any) for human health of this indirect exposure to GM food. Furthermore, the examples in the papers highlighted some differences even between such crude things as macronutrient composition of GM and conventional lines. It is argued by some that these differences have little biological meaning. However, it was clear that most GM and parental line crops would arguably fall short of the definition of 'substantial equivalence'. This crude, poorly defined and unscientific concept outlived its possible previous usefulness. There is an urgent need to come up with novel scientific methodologies to probe into the compositional, nutritional/toxicological and metabolic differences between GM and conventional crops if we want to put this technology on a proper scientific foundation and also to allay the fears of the general public. We need more science and not less. For proper safety assessment, our first concern ought to be to establish on a case-by-case basis the impact of components of GM foods on the digestive system, its structure and metabolism, because the way our body will respond to GM foods will be predetermined at this level. According to the Royal Society (1999), we need 'to refine the experimental design of the research done to date'. New ideas were also advocated in the *Lancet* debate (Ewen and Pusztai, 1999b; Kuiper *et al*., 1999) and at the OECD Conference in Edinburgh in February 2000.

Recommendations

Main tasks and methods for safety assessment of GM crops

1. For compositional analysis and comparison, the parent and transformed lines must be grown under identical conditions, treated and harvested the same way. In addition to proteins, starch, lipids, etc. of the parent and GM lines, their contents of bioactive components should also be compared by novel methods (proteomics, fingerprinting, etc.).

2. The stability to degradation by acid or pepsin or other proteases/hydrolases of GM

products, foreign DNA, including the gene construct, promoter, antibiotic resistance marker gene, etc., has to be established in the stomach and intestines of model animals *in vivo*. With GM lectins, including Bt toxin, the presence/absence of their epithelial binding should also be demonstrated by immunohistology.

3. The biological, immunological, hormonal properties and allergenicity of GM products must be established with the GM product isolated from the GM crop, and not with recombinants from *E. coli*, as these two may have substantially different properties.

4. As GM food is unlikely to be highly poisonous, 'toxicity' is an unhelpful concept and difficult to assay. In contrast, nutritional studies in which GM crop-based diets are fed to young growing animals should reveal their possible harmful effects on metabolism, organ development, immune/endocrine systems and gut flora, which together determine the safety of the GM crop and the development of the young into healthy adults.

5. For animal testing iso-proteinic and iso-energetic diets need to be formulated in which most of the dietary protein is derived from the GM crop. The composition of the control diets should be the same as the GM diet but containing the parent line with or without supplementation with the isolated gene product at the same level as expressed in the GM line. Groups of animals (five or more per group), of similar weight, should be pair-fed in short- and long-term experiments. Urine and faecal samples should be collected for the determination of net protein utilization (NPU), nitrogen balance and feed utilization ratios. Blood samples should be taken before, during and at the end of the experiments for immune studies (i.e. lymphocyte proliferation assay, Elispot), hormone assays (insulin, cholecystokinin, etc.) and for the determination of other blood constituents. The animals are to be weighed daily and any abnormalities observed. After killing the animals, their bodies should be dissected, the gut rinsed and its contents saved for further studies (enzymes, GM products, DNA). Sections should be taken for histology, and the wet and dry weights of organs recorded and analysed.

Evaluation

With suitable statistical analyses (ANOVA, multiple comparisons and/or multivariate analysis), the significance of differences, if any, in the parameters should be established.

- If differences between animals fed GM and parent line diets indicate that the genetic modification must have had a significant effect on utilization and nutritional value, the GM crop cannot be accepted for inclusion in the human/animal diet.
- If, similarly to the GM diet, the parent line diet spiked with the gene product shows differences, the use of this gene in GM food/feed is not acceptable.
- If negative effects are not observed with the parent line diet containing the isolated gene product, it is likely that the harm is caused by the use of the particular construct or by an unwanted or unforeseen effect of the gene insertion on the genome.

Animal testing is but a first step and not a substitute for human studies. If there is no indication of harm to the animals, the results will have to be validated with human volunteers in clinical double-blind, placebo-controlled drug-type tests. Such studies may have to go on for considerable lengths of time. It must also be kept in mind that any potential harm with GM food may be most acute in the young, elderly and sick, particularly those suffering from HIV, hepatitis or other viral diseases. Many people suffer from allergies and other disorders of the gastrointestinal tract, and for these the consumption of GM food may have unforeseen consequences and some of these may be irreversible. Thus, for these, the clear labelling of GM food must be made mandatory.

There is a compelling need to develop further the concepts of biological testing, particularly for potential long-term effects. Since the GM potato work with male rats showed abnormalities in the development of their sexual organs, it is imperative that similar experiments should be done with female rats to be followed by studies of the effects on

reproductive performance of rats (or other animals) reared and maintained on GM vs. non-GM diets for several generations.

If there is a general willingness to fund research along these or similar lines and the regulators accept the concept of biological/toxicological testing transparently and inclusively, the methods are available for the work to start. Following this route, publishing the results and consulting the public will ensure that a technology which promised safe and plentiful food will deliver it for us all, and we are confident that if people see that everything has been done to establish its safety they will accept it willingly.

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