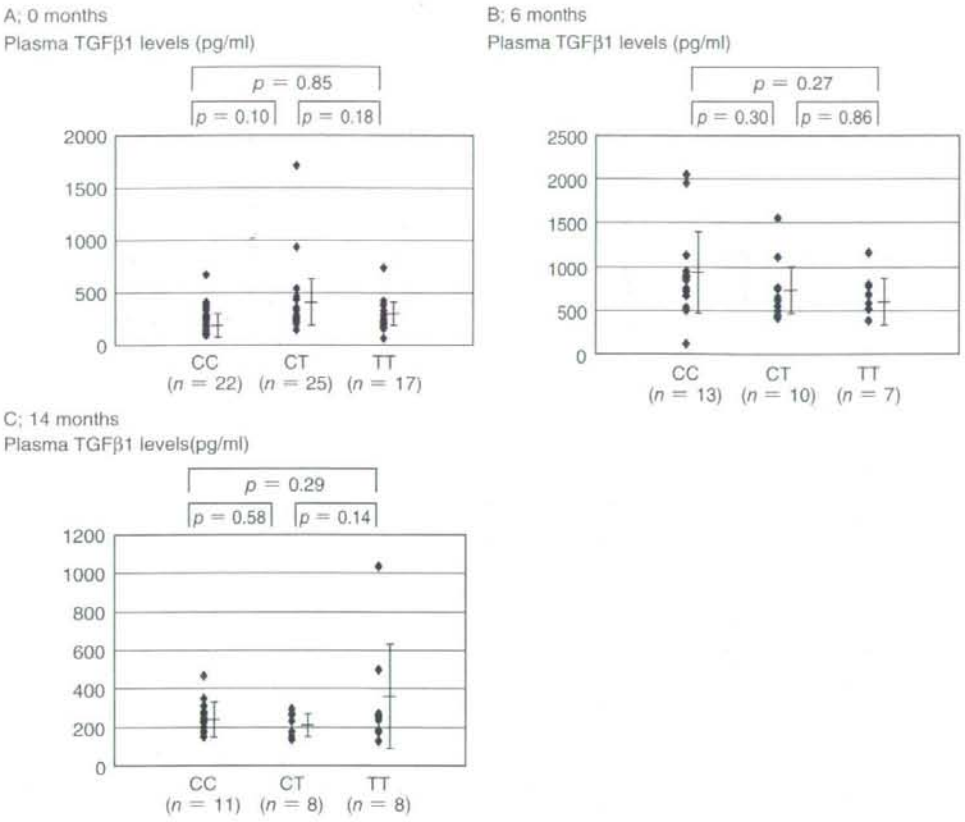

Fig. 3 Relationship between C-509T SNP of TGFβ1 gene and plasma TGFβ1 levels at 0 months (**A**), 6 months (**B**) and 14 months (**C**) is shown.

We also investigated the relationship between plasma IgE levels and TGFβ1 levels at 0, 6 and 14 months of age and found no significant relations (0 months; $p = 0.368$, 6 months; $p = 0.778$, 14 months; $p = 0.818$, data not shown).

PLASMA TGFβ1 LEVELS AND *TGFβ1* C-509T POLYMORPHISM

Among the 64 subjects, 22, 25 and 17 were classified as genotype CC, CT, and TT, respectively. As shown in Figure 3, these genotypes were not significantly related with plasma TGFβ1 levels at either 0, 6, or 14 months of age.

TGFβ1 C-509T POLYMORPHISM AND ALLERGIC DISEASES

The genotypes of *TGFβ1* C-509T polymorphism were not associated with the prevalence of atopic dermatitis at 6 months of age (Table 2) and 14 months of age (Table 3). However, the TT genotype was present in the TGFβ1 gene (C-509T) in all 3 patients with asthma at 14 months of age (Table 4). Furthermore, the TGFβ1 levels (M ± SD; 651.42 ± 544.79 pg/ml) in patients with asthma having the TT genotype in the TGFβ1 gene (C-509T) were higher than the TGFβ1 levels (M ± SD; 243.75 ± 89.19 pg/ml) in subjects without asthma (CC, CT or TT genotypes) at 14 months.

DISCUSSION

TGFβ1 is a multifunctional cytokine that has immunomodulatory effects produced by airway epithelial cells, eosinophils, helper T type 2 lymphocytes, macrophages, and fibroblasts. An important finding in this study is the changing pattern of plasma TGFβ1 levels over time at 0, 6, and 14 months of age. Plasma TGFβ1 levels at 6 months of age were the highest among these 3 measured age points. This pattern was observed in 22 of the 24 subjects whose plasma TGFβ1 levels were available at all 3 points. Statistically, the plasma TGFβ1 level at 6 months of age was significantly higher than that at 0 months and at 14

Table 2 Genotype frequency of C-509T *TGF-β1* promoter SNP in subjects with/without atopic dermatitis at 6 months

	CC	CT	TT	Total
Without atopic dermatitis	18	17	12	47 (74%)
With atopic dermatitis	4	8	5	17 (26%)
				($p = 0.551$)

Table 3 Genotype frequency of C-509T *TGF-β1* promoter SNP in subjects with/without atopic dermatitis at 14 months

	CC	CT	TT	Total
Without atopic dermatitis	18	19	12	49 (77%)
With atopic dermatitis	4	6	5	15 (23%)
				($p = 0.865$)

months of age ($p < 0.0001$ for each). We believe this is the first time this unique change in the plasma TGFβ1 level has been reported.

A previous study showed that the range of plasma TGFβ1 levels was 2000–4000 pg/ml in adult population.[5] In our study, the range of plasma TGFβ1 levels at 0, 6, and 14 months of age was 0–2500 pg/ml, and was lower than that of adult populations. At present, we do not know what causes the plasma TGFβ1 level changes, but one possible explanation could be that TGFβ1 is necessary for dramatic changes in immunological response or maturation at around 6 months of age. In other studies, the CD4 or CD8 cells producing cytokines, including TGFβ1, increased with age.[7,8] An increase in the number of TGFβ1- producing cells may partially explain why TGFβ1 production at 6 months was higher than that at 0 months. We are planning to observe further changes in plasma TGFβ1 levels from 14 months of age to 5 years of age or older.

We could not find any association of IgE with TGFβ1 in this study. TGFβ1 is produced by various cells including airway epithelial cells, eosinophils, lymphocytes, macropharges, and fibroblasts.[9,10] Various cell factors should be considered to evaluate the level of plasma TGFβ1.

It has been hypothesized that the T allele of the C-509T SNP enhances the Yin Yang 1 (YY1) transcription factor consensus binding site (-CCATCTC/TG-) on the TGFβ1 promoter and is responsible for increased TGFβ1 transcription.[11] In the present study there were no significant differences in plasma TGFβ1 levels at 0, 6, and 14 months of age regarding genotypes of *TGFβ1* C-509T. Pulleyn has shown that the T allele of the C-509T SNP is associated with the diagnosis of asthma and asthma severity.[12] In this study, only 3 subjects were given a diagnosis of bronchial asthma by the age of 14 months. Interestingly, the TT genotype was present in these 3 subjects (Table 4, $p = 0.016$). Although the prevalence of bronchial asthma at 14 months of age is low we need to obtain data at 5 years of age for an accurate evaluation of the role of the *TGFβ1* polymorphism in bronchial asthma. We are planning to increase the number of subjects to participate in future studies and to conduct a follow up for a longer time span.

Table 4 Genotype frequency of C-509T *TGF-β1* promoter SNP in subjects with/without bronchial asthma at 14 months

	CC	CT	TT	Total
Without bronchial asthma	22	25	14	61 (96%)
With bronchial asthma	0	0	3	3 (4%)
				($p = 0.016$)

In conclusion, this birth-cohort study suggests that plasma TGFβ1 levels are influenced by age and that the C-509T SNP of the TGFβ1 gene is an important susceptibility locus for asthma in infants at 14 months of age, despite the fact that the number of subjects who participated in this study was limited.

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nature biotechnology

Allergenicity assessment of genetically modified crops—what makes sense?

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GM crops have great potential to improve food quality, increase harvest yields and decrease dependency on certain chemical pesticides. Before entering the market their safety needs to be scrutinized. This includes a detailed analysis of allergenic risks, as the safety of allergic consumers has high priority. However, not all tests currently being applied to assessing allergenicity have a sound scientific basis. Recent events with transgenic crops reveal the fallacy of applying such tests to GM crops.

Genetically modified (GM) crops undergo rigorous safety assessment before being allowed to enter the market. One aspect of GM foods that has drawn a lot of public attention is the assessment of their potential allergenicity. Protecting people with food allergies against accidental exposure to allergens has become an important focus for food manufacturers and regulators responsible for all food safety. A significant focus of the food industry is to keep food products that are not intended to contain a major allergen (e.g., peanut, milk, eggs or wheat) from being contaminated with one of the major allergens. Likewise, the primary focus of the safety assessment for GM crops, as defined by the Codex Alimentarius Commission (Box 1)[1], is to prevent the transfer of a gene encoding a major allergenic protein (from any source), into a food crop that did not previously contain that protein.

The producers of GM crops and regulatory authorities focus on preventing avoidable increases in the risk of allergy in producing and accepting new GM crops. It should, however, be recognized that absolute avoidance of all risk is not achievable. Thus the assessment that has been developed focuses on avoiding risks that are predictable and likely to cause common allergic reactions.

Before discussing the details regarding the approaches used for assessing potential allergenicity of GM crops and the drawbacks of some steps, it is important to put the risks associated with food allergy into perspective. The prevalence of food allergy is not well established but is estimated to be around 6% in young children and 3% in adults[2].

Known potent allergenic foods like peanut or shrimp are not banned from the market, even though 1% of the population might develop allergic reactions upon exposure. In addition, market introductions in the recent past of novel foods like kiwi have resulted in the development of new allergies. Yet kiwi has not been removed from the market. Some of the major allergenic foods like fruits, nuts and fish are considered essential components of a healthy diet, and nobody would endeavor to deprive 99% of the population of these foods because 1% is at risk of developing food allergy. Instead, food labeling is used to help the allergic consumer avoid exposure to foods that cause their reactions. Similar arguments could be made for new crops developed either by conventional breeding or by genetic modification to, for example, help combat malnutrition in developing countries.

Furthermore, to date there is no documented proof that any approved, commercially grown GM crop has caused allergic reactions owing to a transgenically introduced allergenic protein, or that generation of a GM crop has caused a biologically significant increase in endogenous allergenicity of a crop[3]. However, the potential for the transfer of an allergen was illustrated in the 1996 case of transgenic soybeans into which the gene for a 2S albumin from the Brazil nut had been transferred to enhance the methionine content of animal feed. Although the protein had not previously been recognized as an allergen, a study sponsored by the developer of the crop, Pioneer Hi-Bred International (Johnston, IA, USA) during product development demonstrated IgE-binding with sera from Brazil nut–allergic subjects and positive skin prick tests to the transferred protein[4]. This protein is now known as the major allergen of the Brazil nut, Ber e 1. Despite being developed for animal feed only, the product was abandoned because of the obvious risk.

That experience provided guidance for development of the premarket allergenicity assessment process and demonstrated that specific, appropriate tests can prevent the transfer of a gene encoding a protein that might pose substantial risk. However, whereas absolute protection against all potential allergic reactions to a newly introduced protein can never be given, the allergenicity assessment of GM crops based on scientifically sound protocols should minimize the risks. It should be noted that some scientists and regulators have called for postmarket monitoring of GM crops to identify the development of new allergies associated with the crop. The full Codex guidelines[1], however, outlines the need for an effective premarket evaluation as the most effective tool to protect the public. There are technical, practical and economic issues that would need to be addressed in designing an effective postmarket monitoring system and are beyond the scope of this paper. Here, we focus on the scientific validity of protocols used in the premarket evaluation of the potential allergenicity of GM crops. In particular, we show how three tests that are commonly called for, and which have not been validated, can block development of potentially useful products.

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Box 1 Risk assessment of genetically modified crops

The Codex Alimentarius Commission, under the FAO and the WHO, adopted guidelines in 2003 to harmonize the premarket risk assessment process for plants derived from biotechnology (GM plants) in the global market[1]. The guidelines were approved by the Codex Commission and are intended to guide countries in adopting consistent rules that provide a strong food safety evaluation process while avoiding trade barriers. Each new GM crop requires a premarket safety assessment to evaluate intended and unintended changes that might have adverse human health consequences caused by the transfer of the DNA (genes). The goal is to identify hazards, and if found, to require risk assessment and where appropriate develop a risk management strategy (e.g., do not approve, approve with labeling and/or monitoring, or approve without restriction).

The process is based on the science and requires the use of methods and criteria that are demonstrated to be predictive. New methods should be validated and demonstrated to enhance the safety assessment.

The framework to guide evaluation of potential safety issues requires detailed characteristics of:

- The GM plant and its use as food
- The source of the gene
- The inserted DNA and flanking DNA at the insertion site
- The expressed substances (e.g., proteins and any new metabolites that result from the new gene product)
- The potential toxicity and antinutritional properties of new proteins or metabolites
- The introduced protein compared with those known to cause celiac disease if the DNA is from wheat, barley, rye, oats or related grains
- The introduced protein for potential allergenicity
- Key endogenous nutrients and antinutrients including toxins and allergens for potential increases for specific host plants (DNA recipients)

Certain steps in the assessment require scientific assessment of existing information; others require experiments, in which case assay validation, sensitivity and auditable documentation are required

Evolution of guidelines for allergenicity assessment of GM crops

Guidelines for allergenicity assessment of GM crops were published in three sequential documents that have been broadly recognized. The first comprehensive document was published in 1996 by the International Food Biotechnology Council (IFBC, Washington, DC) in collaboration with the International Life Sciences Institute (ILSI, Washington, DC)[5]. This was followed in 2001 by the UN Food and Agriculture Organization (FAO)/World Health Organization (WHO) consultation recommendations[6] and in 2003 by the Codex Alimentarius Commission guidelines[1]. The revised recommendations (FAO/WHO, 2001; Codex, 2003) were meant to correct shortcomings, although further clarifications are possible as we learn more about allergens and gain experience in test methods[7]. Several elements, however, are well established and have remained consistent throughout the three successive sets of recommendations.

All documents agree that introducing known allergens into a different species needs to be avoided as the primary risk is to those with existing allergies. If the source of the gene is a common allergenic food, or if the protein displays significant sequence identity with known allergens, the candidate protein should be evaluated for IgE binding using a sufficient number (e.g., for >95% confidence) of sera from patients allergic to the source of the allergenic food or to the sequence of the matched allergen. Those tests should reveal whether the gene codes for a yet unidentified allergen from a common allergenic source or whether IgE against known allergens cross-reacts with the homologous new transgenic protein. Another parameter included in all three guidelines is resistance of the candidate protein to digestion by pepsin, the rationale being that pepsin resistant food proteins are more prone to induce systemic, severe symptoms. Perhaps more importantly, such stable proteins are also thought to be more potent sensitizers than proteins that are readily digested in the gut (that is, they are risk factors for induction of new allergies).

The IFBC ILSI and FAO/WHO guidelines both used a decision tree to evaluate the risk of allergenicity[5,6], as reviewed previously[7]. The IFBC-ILSI document recommended *in vivo* clinical testing (skin prick tests (SPT) and double-blind placebo-controlled food challenges (DBPCFC)), even if *in vitro* assays had demonstrated a lack of IgE binding for proteins from an allergenic source, if the protein sequence included as little as a single eight amino acid match to a known allergen. Even so, the FAO/WHO recommendations designated *in vivo* clinical testing as impractical and perhaps even unethical under most circumstances as a risk assessment tool, and suggested instead that negative serum testing alone, or in some circumstances SPT testing, but not food challenges, might be necessary to demonstrate a lack of risk. Another change recommended by the FAO/WHO[6] guideline was a six amino acid match to indicate a risk of cross-reactivity with allergens, rather than an eight-amino acid match indicated by IFBC-ILSI[5]. Two additional new elements were added to the FAO/WHO (2001) recommendations: targeted serum screening—in which serum samples of patients allergic (or at least sensitized) to allergen sources broadly related to the source of the gene (sharing similar high taxonomic groups; e.g., monocots, dicots or arthropods) are used to detect or exclude potential cross-reactivity—and animal model testing. Targeted serum screening was recommended even when the transgenic protein did not demonstrate significant sequence identity to a known allergen or when the specific serum screening—using sera from subjects allergic to the source or the sequence-matched allergen—was negative. Animal testing was included despite recognition that validated models predicting risk of sensitization in humans do not (yet) exist.

The Codex Alimentarius Commission guidelines abandoned the risk assessment based on a decision tree and adopted a weight of evidence approach[1]. A decision tree was found to be too rigid in a situation where no single criterion is sufficiently predictive and evidence derived from several types of information, based on tests with different levels of validation, needs to be taken into account. Codex clearly emphasized the need to use scientifically validated testing, specifically removing the demand for nonvalidated animal tests and targeted serum screens and calling for validation of short-sequence matching routines. Instead, a 35% identity over an 80-amino-acid window was recommended as a sufficiently conservative prediction for potential cross-reactivity. These recommendations have not been accepted by some regulators. Clearly, the existence of multiple documents with diverging recommendations coming from different organizations has resulted in confusion and sometimes arbitrary inclusion of tests upon request from regulatory authorities. In some cases, regulators continued to base their judgment on nonvalidated (e.g., animal models) or even rejected (short peptide matches) tests.

Assessment protocols

Here, we look at the scientific soundness of the principles and protocols for allergenicity assessment and present some recent case studies to

illustrate the inappropriateness of nonvalidated methods for allergenicity assessment, whether part of the FAO/WHO[5] recommendations or the Codex[6] guidelines. Figure 1 outlines the Codex guidance's weight-of-evidence approach to evaluate the potential risk of food allergy.

Gene source. The process begins with an evaluation of the source of the gene. If the source of the gene encoding the new protein is a commonly allergenic food (e.g., peanut, hazelnut, hen's egg or cow's milk), a respiratory allergen (e.g., birch or grass pollen or house dust mite) or a contact allergen (latex), IgE-binding studies using sera from patients allergic to the source are required to ensure that the protein encoded by the gene does not bind IgE from those allergic to the source. For serum selection, demographic factors need to be taken into account. Both age and habitat have been shown to influence the molecular recognition profiles of specific IgE (**Box 2**). The number of sera needed is dependent on the degree of confidence considered necessary (largely a political and socioeconomic issue) and the prevalence of recognition of the hypothetical allergen. In other words, do we accept a 5% chance of an allergic reaction in 1% of the population allergic to the source or do we want to be more protective and only accept a 1% chance of a reaction in, for example, 0.01% of that population? Choosing to lower the risk requires a higher number of sera.

If the source of the gene rarely causes allergies, it would be difficult or impossible to find enough qualified serum donors to perform statistically valid tests. However, that also means the number of individuals in the population who would be at immediate risk of reactions if the protein were an allergen would be small. In such cases, the number of individuals is not as important as the specificity of the test and evidence of clinical relevance of the allergenic source.

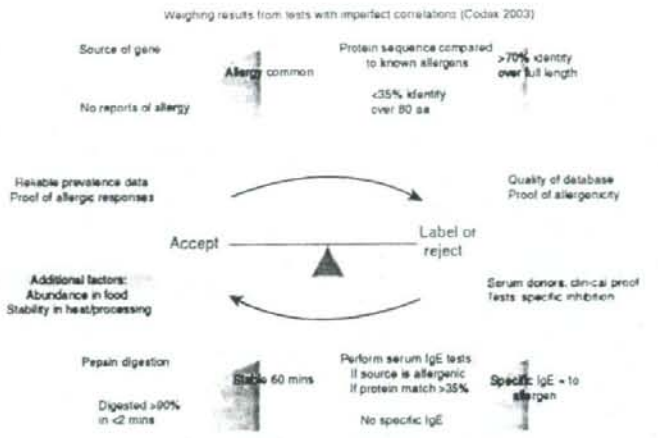

Figure 1 Schematic interpretation of the weight-of-evidence approach described by the Codex Alimentarius Commission Guidelines for Allergenicity Assessment in 2003 (ref. 1). In the figure, the four main areas of evidence are depicted with a graphic representation of the evidence representing maximum risk on the right (high side of the triangles). The weight of the evidence in each of the areas is influenced by the quality of the factors depicted in the yellow boxes. On the basis of the imperfect nature of the test methods available to distinguish between allergenic and nonallergenic proteins, scientific interpretation is necessary to reach a balanced and useful conclusion regarding the potential risks of allergy associated with each new food product.

Bioinformatics. The amino acid sequence of all transferred proteins, regardless of the source, are to be compared with known allergens by FASTA or BLAST algorithms to determine if any identity match is sufficiently high to suspect that the protein might cause allergic cross-reactions. This is not meant to be a stand-alone test, but rather to identify proteins that would require serum testing, using donors with specific allergies to the source of the sequence-matched allergen to evaluate potential IgE binding. If the identity match is high (e.g., >70% over most of the length of the protein), the potential for cross-reactivity is

Box 2 Spaniards are different from Dutchmen

Exposure to allergen is an essential prerequisite for sensitization[47]. An exception to this rule is cross-reactivity: for example, exposure to birch pollen can induce allergy to apple, cherry and hazelnut[27,48,49]. This is typically seen in those areas of the world where birch pollen exposure is high, such as The Netherlands. In the absence of birch pollen, apple allergy also exists, for example, in Spain. In a recent European multicenter study, almost 400 people allergic to apple from four countries were compared to identify potential cross-reactive causes[48]. As expected Dutch, and others (Austrian and Northern Italian) individuals were allergic to apple because they were allergic to birch pollen. IgE binding the major birch pollen allergen Bet v 1 cross-reacted with the homologous major apple allergen Mal d 1. Symptoms induced by Mal d 1 were almost exclusively mild and restricted to the oral mucosa. Spanish participants had not been exposed to birch pollen and were shown to be sensitized to a non-pollen–related allergen identified as a lipid transfer protein (Mal d 3). Although the majority exclusively have mild symptoms in the oral cavity, it was demonstrated that IgE against lipid transfer protein is a significant risk factor for the development of severe systemic symptoms, as were observed in ~25% of the Spanish individuals[48]. This study clearly illustrates that the outcome of allergenicity assessment of GM crops using serum samples of patients with largely identical clinical symptoms upon consumption of apple is strongly influenced by the geographic origin of the patients. Spanish are simply different from Dutch apple-allergic patients due to differences in exposure or other local environmental factors; variations in genetics of these populations cannot account for the marked differences. In the former case, assessment will focus on non-pollen–related apple allergens; in the latter, on birch pollen–related allergens. Similar patterns have been reported for cherry allergy with Pru av 1, a homolog of Bet v 1, being the dominant allergen, compared with lipid transfer protein in cherry, peach and hazelnuts as the primary allergen in the Mediterranean areas[27,49]. These studies highlight the need for good patient characterization and selection before the use of their sera in allergenicity assessment protocols, as differences in the prevalence of IgE sensitivity is possible in the same foods, in different populations.

high and the risk would probably be close to that posed by the matched allergen. Matches sharing between 50% and 70% overall pose a moderate risk of cross-reactivity and should be tested for IgE binding. If the match is <50% identical, the risk of cross reactivity is expected to be low[8]. Even so, a conservative threshold value of 35% identity over any 80 amino acid segment of the transferred protein contained in both the FAO/WHO[6] and Codex documents[1] was intended to identify conserved gene segments representing functional motifs, which might retain conformational epitope structure as well. Proteins with higher matching identities (e.g., >35% identity) are recommended for testing of IgE binding.

On the basis of literature searches, only a few examples of endogenous proteins from sources suspected of cross-reactivity demonstrate significant IgE cross reactivity for proteins sharing between 35% and 50% identity over the entire length of both proteins, and quantitative IgE binding and basophil histamine release (an *ex vivo* test of the circulating effector cells triggered to release histamine by IgE cross-linking) demonstrate only partial reactivity[7]. The lack of known examples of cross reactivity associated with proteins sharing only 35% identity over 80 amino acids suggests the criterion is too conservative as it would overpredict potential cross reactivity. One alternative is to focus on overall sequence alignments, as suggested by Ladics *et al.*[9]. Another alternative would be to increase the percent identity for the 80 amino acid window closer to a level (possibly >50% identity) where there are examples of at least weak *in vitro* cross reactivity using sera from individuals having allergic symptoms to the sources of both proteins[10].

The bioinformatics step is relatively straightforward and should markedly reduce the risk of transferring even a minimally cross reactive protein. However, some allergens that may be matched are rarely noted as causing allergies and it would be virtually impossible to identify appropriate serum donors for a well powered study. In such cases, the risk of potential allergy to the population from that protein is likely to be extremely low and regulators may be willing to waive the requirement for IgE testing. Choosing the appropriate allergen database to search is vital for a reliable sequence comparison[10]. AllergenOnline (http://www.allergenonline.com) is the only database that is currently fully peer reviewed regarding evaluation of published evidence of allergenicity. Other databases are available and the alternative of searching the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) nonredundant database, with key word limits can provide more updated sequences, but lacks an accurate screening method for relevance[10].

Although there are not yet any publications reporting validation of the approach using 35% homology over an 80-amino-acid window (or >50% overall homology) to predict likely cross-reactivity, it is clear that it is an improvement over methods using sequence homology over 6 or 8 amino acids[11]. These short peptide matches have not been validated as predictive tools and should be rejected on the basis of extremely high numbers of false-positive hits[11–14]. The eight-amino acid match was originally selected without evidence of predictability based on the idea that it would represent both a theoretical B cell epitope as well as a minimum size for a conserved T cell epitope[5]. Stadler and Stadler[13] reported that a 6 mer match resulted in more than two thirds of all proteins in Swiss-Prot being predicted to be allergens, and >40% of the human genome being predicted as such. Obviously, the use of short amino matching searches (6–8 mer) is not a useful approach for allergenicity assessment, but it has never been truly renounced. Consequently, a few regulatory authorities sometimes still require bioinformatics analyses based on 6 mer matches (**Box 3**).

Serum IgE binding. Serum IgE testing to evaluate proteins from an allergenic source, or proteins with sequence identity (e.g., >35% over an 80 amino acid widow or >50% overall) to a known allergen works very well if performed properly[15]. Appropriate positive and negative control proteins or extracts of the allergenic source material are required to demonstrate assay validity. The positive test sera must be from clearly diagnosed allergic subjects who react to the gene source or sequence matched allergen and its source. Negative control sera would typically include donors with allergies to other unrelated proteins as well as non allergic subjects. A few relatively well controlled studies have been used to evaluate GM crop safety[4,15,16], although the relevance of donor selection has not always been clear[15].

The design and interpretation of assays for specific IgE can be complex. Potential confounding factors include the molecular appearance of the protein (e.g., monomeric versus multimeric, proper folding or misfolding, presence or absence of disulfide bonds, presence or absence of N linked glycans) and abundance of the protein in the source material (that is, sensitivity). The test material must be representative of the form available in the GM food source. The tests should be capable of detecting IgE binding to linear and conformational epitopes, sometimes requiring two separate assays (e.g., under reducing and native conditions). Demonstration of specificity of binding requires replicate samples with sera exposed to appropriate inhibitors.

Moreover, it must be recognized that there are no absolute thresholds of serum IgE binding that provide absolute measurement of safety or risk. Positive IgE tests without clinical relevance are common in clinical

Box 3 Short peptide match: a lot of work for nothing

Pioneer Hi-Bred International and Dow AgroSciences (Indianapolis, IN, USA) developed a GM maize product containing the gene encoding Cry1F, from *Bacillus thuringiensis*. The product was approved for sale in the United States and Canada following full regulatory studies, including assessment of the potential allergenicity of the protein based on Codex guidelines[1]. The protein produced from this gene is toxic to lepidopteran larval pests, such as the European corn borer, but not to mammals[16]. The gene is from an organism not known to cause allergies. The sequence is not significantly identical to any known allergen based on overall FASTA alignment. It is <35% identical to any 80-amino-acid segment of known allergens, which is the primary alignment criterion recommended by Codex[1]. Because of regulatory requests from Taiwan, an additional bioinformatics comparison was performed to identify any six-amino-acid matches with allergens. There was a single six-amino-acid match to the house dust mite allergen Der p 7 (ref. 16). The protein does not have any other alignment similarity to Der p 7, yet regulators from Taiwan required human allergic serum IgE testing to evaluate potential cross-reactivity. The results of the serum IgE test demonstrated a lack of IgE binding to Cry1F using sera from allergic subjects who had clear IgE binding to Der p 7 (ref. 16). The results satisfied the regulators and the product was approved. However, the tests were expensive and there is always a chance of obtaining a weak-positive IgE binding result. Even the slightest amount of binding would likely have led to extensive *in vivo* testing, but would have been unlikely to demonstrate a risk of an allergic response in consumers as at least two IgE binding sites and high affinity are required to effectively cross-link mast cells and trigger an allergic response (as discussed in reference 11).

practice (e.g., due to the presence of cross reactive IgE to plant N-glycans). To avoid potentially confounding test results, developers may want to remove glycosylation sites before introducing the new gene unless the glycan is needed for functionality. Serum from individuals with strong carbohydrate specific IgE antibodies should be avoided for GM assessment to ensure selection of appropriate donors who should have IgE directed against peptide epitopes rather than carbohydrate. Otherwise, carbohydrate-binding sera would lead to designating most glycoproteins as an allergenic risk, although it is widely accepted that the glycans are unlikely to cause clinical food allergy[17,18]. In the event the transgenic protein is glycosylated, alternative testing may be required to evaluate glycan structure or if IgE binding is demonstrated, the relevance should be tested by basophil histamine release or *in vivo* allergen testing. Diagnosing allergic disease requires a holistic evaluation of diet, symptoms, SPT and/or specific IgE and elimination diet or challenge test[19]. Likewise, interpretation of IgE binding to GM proteins requires judgment. Strong, specific binding to the protein using appropriate donors should be taken as evidence of risk. However, low levels of binding that are not clearly specific and close in affinity to the suspected cross-reactive allergen may not indicate significant risk. If results are equivocal, SPT or challenges might be necessary to demonstrate the relevance of low levels of apparent specific IgE binding.

Stability in pepsin and abundance. The ability of the new protein to withstand digestion by pepsin is evaluated as a potential risk factor of allergenicity[20,21]. Several potent food allergens are known to be very stable in an *in vitro* pepsin digestion assay, whereas it is thought that most dietary proteins are readily digestible[22]. However, some proteins not known to cause significant food allergies are also stable[23]. And some proteins known to cause food allergy, especially those inducing only oral allergy syndrome—mild tingling or itch in the mouth, without substantial edema—are relatively labile[24]. Thus far, food allergens from this last category are mainly found among cross-reactive allergens, where primary sensitization occurs by inhalation (e.g., pollen or latex). These are therefore usually not designated to be 'true' food allergens[25]. Such proteins are likely to pose little risk to consumers if expressed at low abundance in crops.

Some very stable proteins such as thaumatin-like proteins from apple and grape rarely cause allergy or possibly only mild reactions[26], whereas others, like the lipid transfer proteins from a variety of sources, are very stable and may frequently cause severe reactions[26,27]. Some of these stable proteins are inducible pathogenesis-related proteins and expression is variable in foods, which may complicate their recognition as allergens[28]. There is also evidence that some important pepsin labile allergens become more stable with minor shifts in pH (e.g., from pH 2.5 to 2.75 for codfish parvalbumin)[29]. Although the increased stability at moderate stomach pH values may help explain the allergenicity of some of these proteins, the use of standard pepsin stability testing at pH 1.2 or 2.0 still has a good demonstrated predictive value[30].

An additional risk factor for food allergy is the abundance of the protein in food, as many major food allergens account for >1% of the protein in high protein allergenic foods[20]. Others, such as lipid transfer proteins and parvalbumins are less abundant. Abundant, pepsin-stable proteins are more likely to survive digestion in sufficient quantities to facilitate sensitization and become significant food allergens. The typical quantity consumed of specific foods would be expected to have an impact as well, so nonabundant, stable proteins may be potent allergens if a large amount of food is consumed. However, additional scientific data would be required to establish completely objective criteria for acceptance or concern based on stability and abundance. Currently the results are judged relative to common, potent food allergens.

Box 4 Mission impossible: evaluation of changes in endogenous 'hypo-allergenicity'

A transgenic herbicide-tolerant rice, Liberty Link-rice (LLRICE62), was produced by Aventis CropScience (now Bayer CropScience, LP, Research Triangle Park, NC, USA), by inserting the gene for phosphinothricin-*N*-acetyltransferase (PAT) from a bacteria that has not been reported to be allergenic, nor does it share significant sequence identity with any known allergens. The nonglycosylated PAT protein is rapidly digested by pepsin under standard conditions[50]. On the basis of these characteristics, there is no need to test IgE binding to evaluate the potential allergenicity of the PAT protein. US regulators approved the product in 1999 (http://www.agbios.com/dbase.php). However, because rice has been reported (rarely) to cause allergic reactions in humans, the developer performed an *in vitro* IgE binding study of LLRICE62 to compare endogenous allergenicity to a nontransgenic cultivar after their interpretation of the IFBC-ILSI recommendations and based on historical questions from regulatory agencies (pre-1999). Because true (challenge-positive) rice-allergic individuals cannot easily be found, sera of food-allergic subjects with rice-specific serum IgE or skin test–positive reactions to rice extract, or individuals with clinical histories suggestive of rice allergy were used. However, rice-food allergy was not confirmed by food challenge. These individuals were probably sensitized to grass pollen or inhaled rice flour and may be unaffected when ingesting rice based on a paucity of published cases of proven rice allergy and our experiences[51,52].

The unpublished study (personal communication, Donna Mitten, Bayer CropScience, data reviewed by R.E.G.) revealed no significant differences in IgE binding and allergen content between the GM and a genetically similar traditional rice variety. The value of a study based on sera of patients with unconfirmed rice allergy is questionable. Regardless, Canadian authorities approved LLRICE62 in 2006 having been satisfied with the assessment of potential allergenicity that included an evaluation consistent with current guidelines in addition to the results of the serum study (http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/nf-an90decdoc_e.html). It can be argued that it is not justified to evaluate GM crops for potential changes in endogenous allergenicity for a food with extremely low allergenicity because results will generally be meaningless.

However, the story does not end here. Recently, a small amount of seed from a similar GM event (LLRICE601) was discovered in a commercial 'nontransgenic' rice variety. In order to quickly respond to regulatory requests for information about studies of LLRICE601 safety, Bayer CropScience considered the earlier Canadian request for LLRICE62 and decided to perform a similar study if feasible. However, the original sera used for the previous study were no longer available (personal communication, Donna Mitten). Communications with clinical allergists (including M.E., D.H., H.A.S.) in Australia, Japan, Korea, Taiwan and the United States (by R.E.G) indicated the extreme difficulty in obtaining serum donors with clinically defined allergy to rice (as food), and the study was not performed. But, because people who are allergic to rice should avoid eating it, and so few are allergic, it is not clear that there would be any value in performing such a study.

GM crop allergenicity assessment—what is not (yet) useful?

As the assessment of the allergenicity of GM crops has evolved, scientific evaluation of some tests and criteria included in earlier guidance has demonstrated that some specific approaches are not (yet) particularly useful (e.g., six- to eight-amino acid matches, targeted serum tests). Additionally, some new approaches have been espoused that are not sufficiently validated in terms of predicting allergenicity for use in regulatory decision making, although in some cases continued research may be warranted.

Endogenous allergenicity. If a transgene is transferred to a commonly allergenic food, it appears logical to monitor the influence on endogenous allergenicity, which was recommended by the various guidelines on GM crop allergenicity assessment[1,5,6]. These documents, however, have not addressed the level of change that would be (un)acceptable. Several studies have been carried out comparing endogenous allergenicity of nontransgenic and GM varieties. Monsanto (St. Louis, MO, USA) performed an evaluation of herbicide-tolerant soybeans using sera from soy-allergic subjects[31], and also of a potential herbicide-tolerant GM wheat product using sera from ten subjects allergic to wheat. In a comparison of IgE binding, neither study demonstrated any significant differences between the GM crop and non-GM controls (R.E.G., poster presentation, World Allergy Organization meeting, Vancouver, BC, 2003). A study by Lehrer and Reese[32], commissioned by Pioneer Hi-bred International, compared conventional and GM high-oleic acid soybeans using sera from five individuals selected for high IgE binding to soybean extract. A radioallergosorbent-inhibition (RAST-inhibition) assay demonstrated similar IgE binding results between the GM and non-GM varieties. However, what is the risk and what should be done if statistically significant differences are detected?

Serum IgE binding and histamine release were tested in a comparison of ten varieties of Roundup Ready soybean (GM) developed by Monsanto and eight cultivars of non-GM soybean[15]. IgE-inhibition tests demonstrated up to fourfold differences in IgE-binding potencies across both the GM and non-GM varieties, but overall the GM and non-GM varieties were not significantly different. That study illustrated that a head-to-head comparison of a pair of randomly selected soybean varieties may lead to statistically significant differences, even though the apparent allergenicity of the individual varieties falls within the range of responses to several commercially available non-GM soybeans. Apart from the fact that serum samples used in this study originated from subjects that were negative to soy by food challenge (or were not challenged), the variable IgE binding results clearly highlight an aspect that should be taken into account when evaluating effects on endogenous allergenicity: natural variation of allergenicity of available food crops due to differences in the genetics of commercial varieties, and interactions with the environment (e.g., nutrient availability, differences in moisture, temperature, plant pathogens). It is unreasonable to be more stringent toward GM crops with respect to changes in endogenous allergenicity than can already be accounted for by natural variability. Basing judgment on statistical significance alone has no clinical meaning if natural variability is larger. Importantly, the whole discussion about endogenous allergenicity has limited relevance because patients allergic to the food will (should) avoid eating it anyway, GM or not, to avoid allergic reactions.

The soy study results[15] suggest that there is wide variation in IgE binding to different varieties of the same species of non-GM crops, but few studies have been performed to study the question in a systematic way. Various groups have addressed differences in allergenicity between non-GM apple cultivars, focusing on two major apple allergens, the birch pollen-related allergen Mal d 1 and a lipid transfer protein, Mal d 3. Differences in allergenicity have been found by IgE-binding and IgE-inhibition studies, immunoassays for quantifying allergens, *in vitro* basophil histamine release and genomic sequence variability, but also by SPT and DBPCFC as illustrated below.

Sequence variability, possibly translating into differences in allergenicity, has been recently reported for both Mal d 1 and Mal d 3 in different apple cultivars[33]. Most studies focusing on differences in allergenicity of apple cultivars have used IgE based binding (*in vitro* and *in vivo*) as an endpoint. A recent study has evaluated IgE binding and SPT reactivity as well as measuring Mal d 3 content, comparing ten cultivars of apples[34]. The Mal d 3 content varied more than

Box 5 A controversial nonvalidated animal model

A gene encoding an α-amylase inhibitor 1 (αAI) was transferred from kidney bean to field peas to make peas resistant to a bruchid storage beetle[53]. Because of the recommendation for animal model tests by the FAO/WHO[6], the developer tested the product in a mouse model using repetitive intragastric sensitization followed by intratracheal challenge[54]. This model had not previously been used to predict allergenicity of food proteins and we are aware of no other studies that have used an airway challenge or measure of pulmonary cellular infiltration to evaluate food allergenicity. The test results demonstrated stronger eosinophil accumulation in the lungs in mice sensitized and challenged with the GM pea (or αAI from the pea), compared with the kidney bean[54]. This supports increased Th2 inflammation, but not necessarily IgE-mediated allergy. The report described structural differences of the *N*-linked glycan on αAI expressed in peas compared to kidney bean. There was also evidence of different proteolytic processing of the C terminus of the protein. The authors concluded that differences in post-translational proteolytic processing were responsible for the apparent enhanced immunogenicity of the GM product[53].

The mechanism leading to the altered response in mice is not clear, but more importantly, the model has not been widely tested with allergenic and nonallergenic proteins as would seem necessary based on Codex guidelines[1]. In the case of the GM αAI pea, the differences found in glycan structure and protein processing would have been more appropriately investigated by human serum testing to evaluate IgE binding using serum donors with allergies to legumes if regulators wished to have testing beyond the bioinformatics, pepsin digestion and characterization of the protein.

Despite the fact that no scientific evidence was provided for an increased risk of IgE-mediated food allergy in humans, the study aroused a storm of negative publicity for GM crops, being an allergy risk[55]. Although the developer did not report results of a bioinformatics evaluation of the protein, in our hands a FASTA search of AllergenOnline (http://www.AallergenoOnline.com/), version 7.0, revealed one match of 41% identity over an 80-amino-acid segment to peanut agglutinin precursor, a putative allergen. The overall identity was 34.5%. Although this low level match is not likely to indicate cross-reactivity, it is above the Codex criterion. The data suggesting that peanut agglutinin is an allergen should be evaluated in making a final decision on whether to perform human serum-IgE testing, before any regulatory decision to approve the GM crop. In any event, data from a mouse model should not be relied upon to predict allergenicity.

sixfold on a dry material basis across cultivars. The mean wheal area resulting from SPT of the highest Mal d 3 content apple variety (~55 mg/g) was significantly higher (~threefold) than the mean wheal area for the two varieties with lower concentration (~10 mg/g) of Mal d 3. One may infer from the SPT results and Mal d 3 quantities that the cultivar with the highest levels of the allergen (Starking) is probably three times more allergenic on a gram basis than those with lower levels (e.g., Golden Delicious). Similar studies[35] were carried out with 88 apple cultivars focusing on both Mal d 1 and Mal d 3, although not all the results are published (R.v.R., unpublished data). In both cases, differences in allergen content differed up to 100 fold between the extremes, both in allergen quantification and IgE-inhibition assays. Some of these differences had been observed in SPT and DBPCFC testing, with about tenfold differences between individual cultivars. These detailed studies demonstrate the wide range of natural variability of allergenicity in a common non GM food.

Similar tests of soybean varieties by *in vivo* skin reactivity and *in vitro* IgE binding of ten soy cultivars found up to sixfold differences in IgE-binding potencies[36]. Apart from differences between cultivars, natural variability in allergenicity can also occur due to harvest timing and storage conditions[37,38]. Even between individual apples from a single cultivar and harvest, up to tenfold differences in allergenicity have been reported[39]. Yet clinicians and food safety experts do not recommend avoiding certain apple or soybean varieties, nor is there evidence of significant differences in clinical reactivity for the allergic consumer.

Overall, these studies demonstrate the need to establish natural variability of allergenicity of non GM crops before demanding evaluation of changes in endogenous allergenicity of GM crops. Nevertheless, some regulatory authorities have interpreted the guidelines so broadly that they demand evaluation of changes in endogenous allergenicity of foods for which it is virtually impossible to find sufficient truly allergic patients for a well-powered study (**Box 4**).

Of course, in cases where there are specific reasons to suspect a major impact on expression levels of endogenous allergens, special attention has to be given to evaluating allergenicity. This can, for example, be the case when a transcriptional activator is inserted or the transgene is inserted in the coding region for an allergen. Such events should however, not go unnoticed by the developer of a GM crop as detailed molecular characterization of the insert and the protein as well as protein function are required by Codex[1] (**Box 1**).

Targeted serum screens. The FAO/WHO[6] recommendation for broadly targeted serum screens specifically stated that if the source of the transferred gene was a monocotyledonous plant (class Liliopsida), serum should be taken from 50 individuals with allergies to diverse monocot sources (e.g., some allergic to grass pollen, maize, rice or dates) to identify potentially cross-reactive allergens. However, in the Codex guidelines, this was recognized as unlikely to be predictive[1]. There are four or five structural protein families (prolamins, Bet v 1-relatives, cupins and profilins) with representative clinically cross-reactive allergens from taxonomically diverse sources[40]. Although a few individuals react to material from sources as diverse as representatives of an order (e.g., Fabales) or even higher group, most clinically important cross reactions are elicited by material from within the taxonomic family (e.g., Fabaceae), tribe (e.g., Phaseoleae) or, more commonly, genus (e.g., *Phaseolus*)[41]. Differentiating between clinical cross reactivity, cosensitization and irrelevant IgE binding (low affinity or binding to cross-reactive carbohydrate determinants) is often complicated as clinical reactivity is rarely measured, rather some level of skin prick sensitivity or direct *in vitro* IgE binding is used to define cross-reactivity and this is likely to overestimate clinical reactivity[10,42,43]. Although validated specific serum tests with samples from clinically well characterized subjects allergic to the source of the gene—or allergic to a sequence matched allergen—should be useful when the need is indicated, targeted testing is unlikely to provide reliable data for the assessment.

Animal models. The FAO/WHO[6] recommendations called for evaluating each new GM crop with studies in two separate species of animals and/or using two routes of sensitization in one species, even though the panel recognized that no current animal model is predictive of allergenicity in humans. There are still no validated animal models for predicting allergenicity to food proteins, even though many models have been successfully applied to dissect mechanisms of allergic responses and potential changes due to modification of the allergenic proteins[44,45]. Even though many authors recognize that different animal models respond to specific proteins differently[46], they still suggest using animal models in the safety evaluation process for GM crops. On the basis of the paucity of correlative data between any one animal model and human food allergenicity, and the complex genetic diversity that predisposes subjects to allergy, it is not clear that any animal model could be useful in predicting the potential allergenicity in humans of a novel protein or GM crop. It is also not clear how one might combine results from two animal model tests to produce a predictive result. An unpublished study coordinated by the ILSI-Health and Environmental Sciences Institute (Washington, DC) reported results from a multi-laboratory test of the most commonly used mouse strains (BALB/c, C3H/HeJ, A/J and BDF 1) using commonly recommended protocols to evaluate IgE and allergic responses to identical samples of common potent allergens of peanut (Ara h 1 and Ara h 2) and milk (beta-lactoglobulin) compared with relatively nonallergenic proteins of spinach (RUBISCO) and soybean (lipoxygenase). The responses to the potent allergens were equivalent or weaker than responses to the weakly or nonallergenic proteins (Thomas, K. *et al.*, 2005 annual meeting poster, American Academy of Allergy Asthma and Clinical Immunology).

Box 6 Balb/c mice no substitute for human IgE recognition evaluation

A gene encoding amarantin was transferred from *Amaranthus hypochondriacus* into maize[56]. Although the protein was digested in the pepsin assay, comparing the sequence to known allergens identified a number of 6-, 7- and 8-amino-acid matches to known allergens[56]. Although noting overall homology to some allergenic proteins, the developer decided to use animal models to evaluate the allergenicity of the GM maize[56]. Comparing the amarantin sequence by FASTA demonstrated up to 70% identity over an 80-amino-acid segment to known allergens and >40% identity for overall alignments to a number of important 11S globulin allergens[7]. Clearly, this should have set off an alarm calling for serum IgE testing, if not immediately convincing the developer that the protein was too risky to transfer. Instead, the immunogenicity of the product was tested in BALB/c mice, with results demonstrating no significant response and the authors suggested there was no significant risk of allergy[56]. Although it is not clear if this potential product has been submitted for regulatory review anywhere, the Codex guidelines (2003) indicate that the amarantin-containing maize would require serum IgE testing with sera from at least a number of buckwheat- and/or Brazil nut–allergic subjects and possibly others.

On the basis of current knowledge, therefore, we recommend continuing research to evaluate potentially predictive animal models but caution against testing potential products at this time as there is no scientific validation demonstrating predictive values that are acceptable for risk evaluation. This opinion is also reflected in the recommendations of the Codex Alimentarius Commission[1]. Producers as well as regulators are sometimes confused about which recommendations to follow. This is illustrated by two cases in which developers of GM crops used animal models to evaluate potential allergenicity. In one, case results were interpreted as demonstrating likely allergenicity of the GM crop (**Box 5**) and in another case to suggest absence of allergenicity, even though there is a strong potential for cross-reactivity based on bioinformatics (**Box 6**). In either case, there is no scientific justification for these conclusions.

Conclusions

The current safety assessment outlined in the Codex guidelines (2003)[1] is based on the current state of knowledge regarding food allergens and risk, and is therefore well suited to evaluate the potential for increased risk in allergenicity of GM crops compared with the risk of allergy from the conventionally bred crop varieties. The weight-of-evidence approach was adopted in part as it was recognized that there are exceptions to each component in the process. Thus, each product must be reviewed on a case-by-case basis and experienced scientists must be able to interpret results in aggregate. Key elements of this weight-of-evidence assessment are illustrated in **Figure 1**:

- Source of the gene: common allergen or not?
- Bioinformatics: sequence searches for matches of >35% identity over 80 amino acids (or of >50% overall identity for more realistic risks).
- IgE testing: does the introduced protein bind IgE antibodies?
- Stability testing: is the expressed protein highly resistant to digestion by pepsin?
- Abundance: is the protein abundant in the food (and stable)?

The premarket assessment recommended by Codex provides a mechanism to intercept GM crops that are likely to increase the risk of food allergy, as demonstrated by the identification of the Brazil nut 2S albumin transferred to maize, and the amarantin transferred to maize (**Box 6**) as proteins that would likely present significant health risks for specific populations of allergic consumers. The premarket screening process helps to avoid possible severe reactions in unsuspecting allergic consumers and also prevents subsequent costly food and seed recalls that would be needed to prevent additional reactions.

There is no scientific justification for inclusion of the following tests in allergenicity assessment because their predictive values have not been validated:

- Bioinformatics: short peptide matches resulting in random false positive hits.
- Animal models: useful for mechanistic studies but not applicable for prediction of human sensitization to food.
- Endogenous allergenicity: natural variability needs to be taken into account first.
- Targeted serum screens: potentially high rate of false positive and low probability of true positive results.

Demanding inclusion of such nonvalidated tests can lead to the rejection of safe and beneficial products, excessive costs and, potentially, disruption of trade without any further reduction of risk. Importantly, the use of inappropriate tests such as unvalidated animal models in place of more appropriate tests could lead to the introduction of a product that does pose substantial risk for a group of allergic consumers.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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Functional Analysis of the Thymic Stromal Lymphopoietin Variants in Human Bronchial Epithelial Cells

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Thymic stromal lymphopoietin (TSLP) is an IL-7–like cytokine that triggers dendritic cell–mediated T helper (Th)2 inflammatory responses, and is implicated in the pathogenesis of allergic diseases in humans. Two *TSLP* splice variants have been reported. To find functional genetic variants that might contribute to disease, we conducted analyses of single nucleotide polymorphisms (SNPs) of the *TSLP* gene in human bronchial epithelial cells. We surveyed SNPs on the *TSLP* gene by sequencing genomic DNA from 36 subjects, and characterized the linkage disequilibrium of the gene. We examined whether the SNPs have functional effects on mRNA expression or protein production using real-time PCR, reporter gene analysis, and enzyme-linked immunosorbent assay. We identified a total of 23 polymorphisms in the *TSLP* gene. The long form of *TSLP*, which is associated with allergic inflammation, was highly induced by poly(I:C) (double-stranded RNA) stimulation in normal human bronchial epithelial cells (NHBE) ($P = 0.0060$). The SNP rs3806933 (−847C > T) in the promoter region of long-form *TSLP* was found to create a binding site for the transcription factor activating protein (AP)-1, and *in vitro* functional analyses demonstrated that the SNP enhanced AP-1 binding to the regulatory element. The functional variant increased promoter-reporter activity of long-form *TSLP* in response to poly(I:C) stimulation in NHBE. Functional genetic polymorphism of the *TSLP* gene appears to contribute to Th2-polarized immunity through higher TSLP production by bronchial epithelial cells in response to viral respiratory infections.

Keywords: bronchial epithelial cells; dsRNA; polymorphisms; splicing variants; *TSLP*

CLINICAL RELEVANCE

We identified a single nucleotide polymorphism that creates a binding site for activating protein-1 and affects the transcriptional efficiency of the long-form TSLP induced by poly(I:C) in bronchial epithelial cells. The variant may be involved in the pathogenesis of T helper (Th)2-mediated diseases.

Dendritic cells (DCs) play a crucial role in the pathogenesis of allergic diseases, and thymic stromal lymphopoietin (TSLP) activates $CD11c^+$ DCs and induces production of T helper (Th)2-attracting chemokines (1–3). TSLP-activated DCs prime naïve T cells to produce the inflammatory cytokines IL-4, IL-5, and IL-13, while down-regulating IFN-γ and IL-10 (2–5). Recent murine studies have shown a central role for TSLP in the development of allergic asthma (6, 7). Lung-specific expression of a *TSLP* transgene induces allergic airway inflammation characterized by infiltration of Th2 cells, goblet cell hyperplasia and increased serum immunoglobulin (Ig)E levels (6). *TSLP receptor* (*TSLPR*) knockout (KO) mice exhibit strong Th1 responses, with high levels of IL-12 and IFN-γ, but low production of IL-4, -5, -10, -13 and IgE (7). Furthermore, *TSLPR* KO mice failed to develop an inflammatory lung response to an inhaled antigen (7). In humans, epithelial cells trigger DC-mediated inflammation by producing TSLP in allergic diseases (1), and *TSLP* expression in asthmatic airways is increased and correlated with both the expression of Th2-attracting chemokines and disease severity (8). These findings prompted us to search for a functional *TSLP* polymorphism as a candidate genetic factor for involvement in respiratory diseases such as asthma.

Recent studies have shown higher expression of *TSLP* mRNA at host environmental interfaces such as human primary skin keratinocytes, bronchial epithelial cells, and lung fibroblasts (1, 2). Respiratory viral infections can influence both the development and severity of asthma, and frequently cause acute exacerbation of the disease (9, 10). However, the cellular and molecular mechanisms of the interactions between viral infection and allergic inflammation remain unclear (10). The epithelial cell is a first defense line in the pathogenesis of viral respiratory infections, which can initiate innate immune responses (9). Toll-like receptor (TLR)3 has been shown to recognize double-stranded (ds)RNA and mediate antiviral activity against rhinovirus infection of human bronchial epithelial cells (11–13). dsRNA produced by RNA viruses such as rhinoviruses and respiratory syncytial viruses during replication in infected cells is a potent stimulus for innate antiviral immune responses, and

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polyriboinosinic:polyribocytidylic acid [poly(I:C)] is thought to mimic the effects of dsRNA (14). Airway epithelial cells play an essential role in the innate host defense against infection (15) and highly express TLR2, TLR3, TLR5, and TLR6 (16). It was only recently found that the inflammatory mediators IL-1β and TNF-α regulate human *TSLP* gene expression, and human *TSLP* mRNA levels also increase after exposure to TLR2, TLR3, TLR8, and TLR9 ligands in airway bronchial epithelial cells (17, 18). Another study has shown that TSLP is released by small airway epithelial cells in response to bacterial peptideglycan, lipoteichoic acid, and poly(I:C) (19).

In this study, we examined mRNA expression of two *TSLP* splicing variants separately, and found that the long form of *TSLP* was highly induced by poly(I:C) stimulation in normal human bronchial epithelial cells (NHBE). We also found that a promoter polymorphism (rs3806933) created a binding site for the transcription factor activating protein (AP)-1 and enhanced AP-1 binding to the regulatory element. The functional variant also increased promoter-reporter activity of long-form *TSLP* in response to poly(I:C) stimulation in NHBE.

MATERIALS AND METHODS

Additional details are provided in the online supplement.

Screening for Polymorphisms

To identify single nucleotide polymorphisms (SNPs) in the human *TSLP* gene, we sequenced all exons, including 4.1 kb of the 5′ flanking region and a 1-kb continuous 3′ flanking region of the last exon, except for regions with interspersed repeats, from 24 subjects with asthma and 12 control volunteers. The 16 primer sets listed in the online supplement (*see* Table E1) were designed on the basis of genomic sequences from the GenBank database (accession number AC008572.6). Sequences were assembled and polymorphisms were identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI).

Statistical Analysis

Pairwise linkage disequilibrium (LD) was calculated as r^2 by using Haploview 3.2 (Massachusetts Institute of Technology, Cambridge, MA; http://www.broad.mit.edu/mpg/haploview/). Genotype distribution among 36 subjects in this study was compared with genotype frequencies in the public JSNP 550typed database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/) and the International HapMap project (http://www.hapmap.org/index.html.en), by the contingency χ^2-test. Comparisons in reporter assays, mRNA expression analysis, and protein expression analysis were performed with Student's *t* test or the Mann-Whitney U-test. Statistical significance was defined at the standard 5% level.

Cells, Reagents, and Stimulation

NHBE, normal human lung fibroblasts (NHLF), and bronchial smooth muscle cells (BSMC) were purchased and maintained using medium kits (BulletKit; Cambrex, East Rutherford, NJ). IL-1β, TNF-α, IL-4, and IL-13 were purchased from Peprotech EC, Ltd. (London, UK). Cells were stimulated with 10 μg/ml poly(I:C) (InvivoGen, La Jolla, CA), 100 ng/ml lipopolysaccharide (LPS) (InvivoGen), 1 μg/ml macrophage-activating lipopeptide (MALP)-2 (Alexis, Lausen, Switzerland), 10 ng/ml IL-1β, 10 ng /ml TNF-α, 100 ng/ml IL-4, and/or IL-13. RNaseA was purchased from Roche Diagnostics (Basel, Switzerland). Phosphate-buffered saline (PBS) was used as the vehicle.

Quantitative Real-Time RT-PCR and Enzyme-Linked Immunosorbent Assay

The expression of *TSLP* was determined by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) using SYBR Premix Ex Taq (Takara, Shiga, Japan). In all experiments, the amounts of cDNA were standardized by quantification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). TSLP in culture supernatants was measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., Minneapolis, MN). The mean lower detection limit of the ELISA kit was approximately 31.3 pg/ml according to the manufacturer's protocol. However, in the present work, we calculated the lower detection limit from the standard curve, and the sensitivity of the TSLP ELISA kit was 15.6 pg/ml.

Determination of Transcriptional Initiation Sites

To determine the 5′ arrangement of the *TSLP* mRNA, 5′-RACE was performed by using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA). We extracted total RNA from poly(I:C)-activated NHBE and used it for SMART cDNA synthesis. We obtained the major PCR products, and after isolation and subcloning, eight clones were identified and sequenced.

Luciferase Assay

The promoter and last exon fragments from human genomic DNA were amplified by PCR. PCR products were subcloned into the reporter gene pGL3-promoter vector (Promega, Madison, WI). TRANSFAC Professional 10.3 (http://www.biobase.de/pages/) was used to predict putative transcription factor-binding sites. To confirm AP-1–mediated promoter activity of the long-form *TSLP*, NHBE were transiently transfected with a *TSLP* long-form promoter-driven luciferase reporter plasmid with expression vectors for AP-1 (*c-jun* and *c-fos*) or a mock vector.

Electrophoretic Mobility Shift Assay and Biotinylated Oligonucleotide Precipitation Assay

We prepared nuclear extracts from NHBE as previously described (17). Electrophoretic mobility shift assays (EMSAs) were performed using Gel Shift Assay Systems (Promega). Binding affinity of transcription factors to oligonucleotides was measured *in vitro*. NHBE cell lysates interacting with the oligonucleotides were precipitated by avidin-sepharose, and the bound proteins were separated on SDS-polyacrylamide gels. AP-1 was detected by immunoblotting with anti–AP-1 antibodies (Ab-2) (Oncogene Research Products, San Diego, CA).

RESULTS

Identification of Genetic Polymorphisms in the *TSLP* Gene and Linkage Disequilibrium

We found 23 genetic variants in the *TSLP* region by resequencing DNA samples from the 36 subjects. A total of seven polymorphisms had estimated minor allele frequencies (MAF) of greater than 5% (Table 1), and we calculated r^2 as the statistical value for pairwise LD among these seven variants (Table E2). Allele frequencies of SNPs based on the sequencing of the 36 DNA samples did not differ from those in the general Japanese population, which were obtained from the public JSNP 550typed database and International HapMap project (Table E3) (data not shown).

The SNPs rs3806932, rs3806933, rs2289276, rs11466741, rs2289277, and rs10073816 were in strong LD ($r^2 \geq 0.87$), and most of them were located in the putative promoter regulatory regions of the two splice variants (Figures 1A and 1B). We next estimated the frequencies of the haplotypes and identified three common haplotypes in the 36 subjects (Figure 1C), covering more than 97% of the population.

Poly(I:C)-Induced Expression of Long Splice Form of *TSLP* in NHBE

The gene *TSLP* contains four exons, and a public database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) noted two alternative splice variants of *TSLP*: short-form and long-form *TSLP*. The splice variants use different initiation methionine codons for protein translation. Recent expression studies did not examine mRNA expression of the two splicing variants separately (17–19). We investigated whether the splice variants

TABLE 1. LOCATIONS AND ALLELE FREQUENCIES OF POLYMORPHISMS IN *TSLP*

SNP No.		SNP Long Form*	Location Long Form	SNP Short Form*	Location Short Form	Allele Frequency (%)	rs No.†
1	A > G	−3693	5′ flanking site	−5394	5′ flanking site	1	13186909
2	AGTG > del	−3641	5′ flanking site	−5342	5′ flanking site	1	—
3	T > A	−3492	5′ flanking site	−5193	5′ flanking site	1	—
4	G > A	−3404	5′ flanking site	−5105	5′ flanking site	3	17551370
5	A > C	−2590	5′ flanking site	−4291	5′ flanking site	4	10455025
6	A > G	−1914	5′ flanking site	−3615	5′ flanking site	29	3806932
7	A > G	−1070	5′ flanking site	−2771	5′ flanking site	1	—
8	C > T	−847	5′ flanking site	−2548	5′ flanking site	29	3806933
9	C > T	−82	exon 1	−1783	5′ flanking site	26	2289276
10	C > T	414	intron 1	−1288	5′ flanking site	4	1898671
11	C > A	591	intron 1	−1111	5′ flanking site	3	10062929
12	C > T	1117	intron 2	−585	5′ flanking site	26	11466741
13	G > A	1164	intron 2	−538	5′ flanking site	1	—
14	C > G	1479	intron 2	−223	exon 1	29	2289277
15*	C > G	1560	intron 2	−142	exon 1	22	2289278
16	C > T	1908	intron 3	207	intron 1	3	—
17	A > C	4403	exon 4	2702	exon 2	1	—
18	G > A	4740	exon 4	3039	exon 2	1	—
19	A > G	4997	exon 4	3296	exon 2	3	11466749
20	G > A	5306	exon 4	3605	exon 2	3	11466750
21	G > A	5901	exon 4	4200	exon 2	29	10073816
22	C > G	6143	3′ flanking site	4442	3′ flanking site	3	11466754
23	A > C	6339	3′ flanking site	4638	3′ flanking site	3	—

Definition of abbreviations: SNP, single nucleotide polymorphism; TSLP, thymic stromal lymphopoietin.

* Numbering according to the genomic sequence of *TSLP* (accession number AC008572.6). Position 1 is the A of the initiation codon.

† Number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).

of *TSLP* mRNA were up-regulated by dsRNA and other TLR ligands separately in cultured NHBE, NHLF, and BSMC by examining expression patterns of variants by isoform-specific real-time PCR using cDNA panels of various human tissues (Figure E1A). The long splice form of *TSLP* was not expressed in every examined tissue, whereas the expression of the short splice form was consistently observed in all of the tissues (Figure E1A). We next investigated the *TSLP* expression in lung-derived primary cells, including NHBE, NHLF, and BSMC (Figure 2A). Although the short form of *TSLP* was expressed in NHBE, NHLF, and BSMC, the long form of *TSLP* was not spontaneously detected in NHBE and was barely expressed in NHLF and BSMC (Figure 2A). Respiratory infections are well recognized as major triggers of exacerbation of asthma in children and adults (9, 10). We further assessed whether the long form of *TSLP* could be induced by some pathogen-associated microbial patterns recognized by TLRs and other pattern-recognition receptors. In NHBE, the long form was strongly and temporarily induced within 4 hours by stimulation with poly(I:C) but not by LPS or macrophage-

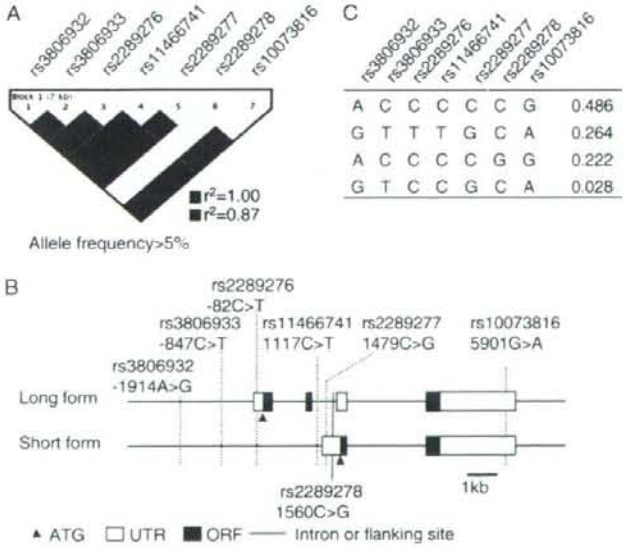

Figure 1. Single nucleotide polymorphisms (SNPs) and pairwise linkage disequilibrium (LD) map of the *thymic stromal lymphopoietin* (*TSLP*) gene. (*A*) LD structure at the *TSLP* locus using SNPs with allele frequencies greater than 5%. Pairwise r^2 values for all combinations of SNP pairs are shown in *grayscale*. (*B*) A graphic overview of polymorphisms with allele frequencies greater than 5% identified in relation to the exon/intron structure of the human *TSLP* gene. The translation start site (ATG), untranslated region (UTR), and open reading frame (ORF) are shown by *solid triangles*, *open boxes*, and *solid boxes*, respectively. (*C*) Haplotype frequencies for *TSLP* SNPs.

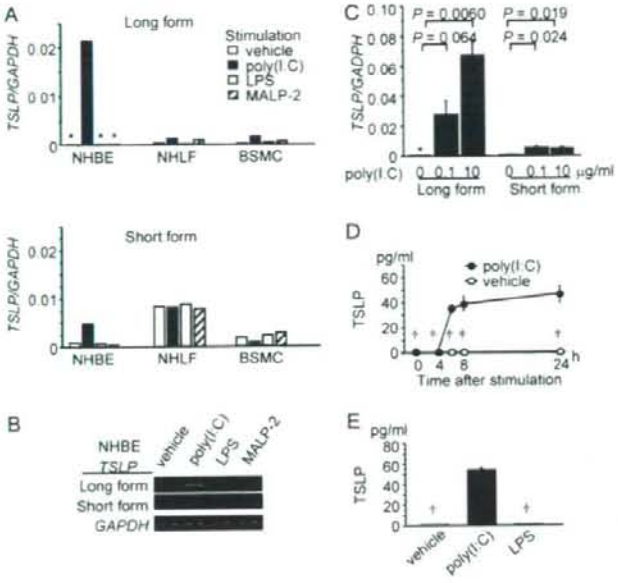

Figure 2. Induction of *TSLP* expression and TSLP protein production in normal human bronchial epithelial cells (NHBE) in response to pathogen-associated microbial patterns. (*A*) Quantitative RT-PCR assay of the long and short splice forms of *TSLP*. NHBE, normal human lung fibroblasts (NHLF), and bronchial smooth muscle cells (BSMC) were stimulated for 4 hours. PBS was used as vehicle. * Not detectable. (*B*) Representative agarose gel showing RT-PCR products from the long and short splice forms of *TSLP* in NHBE stimulated for 4 hours. (*C*) NHBE derived from four individuals were stimulated with indicated doses of poly(I:C) for 4 hours. The expression levels were normalized with *GAPDH* expression. Data represent mean ± SEM of duplicate samples and are representative of two independent experiments. *P* values were obtained by Student's *t* test. (*D* and *E*) ELISA of TSLP in culture supernatants of NHBE stimulated for the indicated times (*D*), and for 24 hours (*E*). A dagger (†) indicates that the concentration was below the detection threshold (< 15.6 pg/ml). Data represent mean ± SD of triplicate samples.

activating lipopeptide 2 (MALP-2), and the short splice form was scarcely induced (Figures 2A, 2B, and E1B). We confirmed the significant induction of the long splice form after stimulation with poly(I:C) using NHBE derived from four individuals (Figures 2C and E1C), and treatment of the poly(I:C) preparation with RNaseA abolished the *TSLP* induction (Figure E1D). The genotypes and Ct values compared with *GAPDH* of four individuals are shown in Table E4.

Recent studies have shown that proinflammatory and Th2 cytokines play important roles in the induction of *TSLP* expression by bronchial epithelial cells or keratinocytes during allergic inflammation (19, 20). We next examined whether treatment of NHBE cells with proinflammatory cytokines such as IL-1β or TNF-α would enhance the long-form *TSLP* gene expression. The long form of *TSLP* was induced by IL-1β and TNF-α in NHBE cells; however, the induction levels were lower than that by poly(I:C) (Figure E2A). Neither of these cytokines influenced the expression level of short-form *TSLP* (Figure E2A). We further examined effects of Th2 cytokines on the *TSLP* mRNA expression, and IL-4 and IL-13 synergistically enhanced the long-form expression of the *TSLP* gene by NHBE cells in response to poly(I:C) (Figure E2B). Expression of short-form *TSLP* was not induced by these Th2 cytokines (Figure E2B). The results imply the importance of the regulation of long-form *TSLP* expression in patients during allergic inflammation.

To examine the effect of the induction of mRNA expression of *TSLP* in NHBE, we further measured TSLP protein products in the culture supernatant of NHBE by ELISA, and found that the TSLP protein was upregulated within 6 hours after poly(I:C) stimulation (Figure 2D). However, no protein induction of TSLP was detected after LPS stimulation (Figure 2E).

Determination of the Transcriptional Start Sites of the Long and Short Forms of *TSLP*

To identify the transcriptional start sites of the two splicing forms of the *TSLP* gene, we performed a rapid amplification of cDNA ends (RACE) procedure using mRNA from NHBE cells. The locations of the *TSLP*-specific primers used in 5′-RACE cDNA amplification for the long and short splice forms are indicated in Figure E3A. The major products were obtained by using long- and short-form-specific primers, respectively (Figure E3B). The 5′-ends of these clones are shown in Figure E3C. In these clones, we could not find any novel exon.

Regulatory Effect of SNP on Expression of the Long Splice Form of *TSLP* in Response to poly(I:C)

To clarify whether the SNPs in the *TSLP* promoter region affected the expression of the two splice variants, we constructed plasmid clones containing the NF-κB regulatory region, which has been previously reported, promoter SNPs, and the exon 1 SNP of the short- and long-form *TSLP* genes. Each construct included a possible combination of the promoter and exon 1 SNPs of the two splice variants and a luciferase gene transcriptional unit in the 5′-to-3′ direction (-1914A-847C-82C [Major] and -1914G-847T-82T [minor] for the long form, and -3615A-2548C-1783C-585C-223C [Major] and -3615G-2548T-1783T-585T-223G [minor] for the short-form) (Figure 3A). The effects of the promoter sequences were examined in NHBE. The reporter activity of the clones containing the promoter and exon 1 SNPs of long-form *TSLP* was enhanced by stimulation with poly(I:C), and the clone containing the -1914G-847T-82T [minor] haplotype showed significantly greater transcriptional activity than the other haplotype, -1914A-847C-82C [Major] (Figure 3A). In contrast, the reporter activities of clones of short-form *TSLP* had no effect on transcriptional activity under culture conditions with and without poly(I:C) (Figure 3A). These results implied that the SNP in the promoter region of the long form of *TSLP* was able to enhance the poly(I:C)-dependent transcriptional activity. We next conducted deletion construct work to verify that the region studied actually contained the important transcriptional regions for the long-form *TSLP* gene (Figure 3B). The deletion construct also had transcriptional activity. We also found that

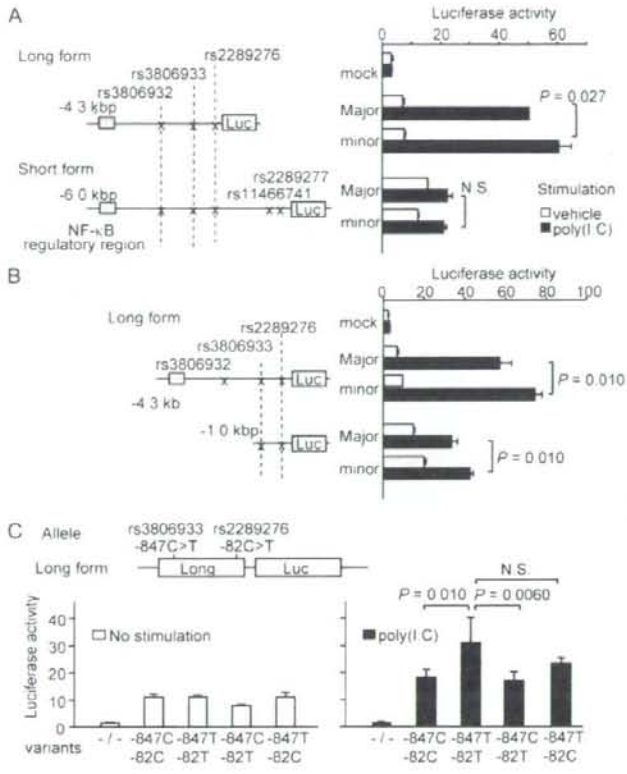

Figure 3. Splicing form- and allele-dependent transcriptional regulation of *TSLP* in NHBE stimulated with or without poly(I:C) for 4 hours. Data represent mean ± SD. (*A*) Transcriptional regulatory activities of haplotypes of long and short splice forms of *TSLP* in NHBE. The *gray box* indicates the NF-κB regulatory region. Data from three experiments in triplicate. $P = 0.027$, by Student's *t* test. (*B*) Identification of effects of SNPs in promoter region of long-form *TSLP* gene on the transcriptional activities. Data from three experiments in triplicate. $P = 0.010$ and $P = 0.010$, by Student's *t* test. (*C*) Schematic representation of the *TSLP* reporter constructs and relative luciferase activities of four haplotypes of the long splice form of *TSLP*. $P = 0.010$ and $P = 0.0060$, by Student's *t* test. Representative data from three experiments done six times.

the clone containing the -847T-82T [minor] haplotype showed significantly greater transcriptional activity than the other haplotype, -847C-82C [Major] in both clones with and without the NF-κB regulatory region (Figure 3B).

To identify the critical SNP responsible for the difference in transcriptional activity of the long splice form, we generated two other constructs, -847C-82T and -847T-82C (Figure 3C). The effects on transcriptional activity were examined in NHBE with and without poly(I:C) stimulation. No obvious transcriptional activity in these constructs was noted without stimulation, whereas, after poly(I:C) stimulation, C base substitution for the -847T base on the minor allele (-847T-82T to -847C-82T) resulted in significantly impaired transcriptional activity of the long splice form (Figure 3C). The impaired activity of the -847C-82T fragment was similar to that of the major allele (-847C-82C) (Figure 3C). These results suggested that -847C > T (rs3806933) played a functional role in transcriptional regulation of the long form of *TSLP*.

To assess the possibility of a regulatory role of the 3′-untranslated region (UTR) SNP in the expression level of *TSLP*, we examined the effect of the SNP on the stability of *TSLP* mRNA (Figure E4A). There was no difference among the stability profiles of the 3′-UTR SNP (rs10073816) in response to poly(I:C) stimulation (Figure E4B).

Transcription Factor Binding to the Regulatory SNP

We subsequently looked for nuclear factors that might bind to oligonucleotides corresponding to the genomic sequences of the promoter alleles of *TSLP*. We predicted a potential allelic difference in the cis-acting regulatory function in transcription by a bioinformatics approach (TRANSFAC). The sequence containing the -847T SNP (rs3806933) on the promoter region created a new consensus sequence corresponding to the putative binding element to AP-1 (Figure 4A).

Nuclear factor-kappa B (NF-κB) and AP-1 are transcription factors crucial for inducing the expression of various cytokines dependent on TLR3 (21, 22). Using nuclear extracts from NHBE stimulated with poly(I:C), we observed nuclear transfer of NF-κB and AP-1 within 1 h by EMSA (Figure 4B). We next examined the binding of AP-1 protein to the sequences containing the -847C > T (rs3806933) by a precipitation assay. Significant binding of AP-1 protein to the -847T oligonucleotide was detected in response to poly(I:C) stimulation; however, only faint binding activity to -847C oligonucleotides was seen with or without poly(I:C) stimulation (Figure 4C). Since the activation of total nuclear AP-1 protein without stimulation was low (Figures 4B and 4C), it appeared that the binding of AP-1 to -847T oligonucleotides was dependent on the activation of AP-1 protein. To further elucidate how AP-1 was involved in the critical SNP responsible for the difference in transcriptional activity of the long splice form, we analyzed the effects of AP-1 overexpression on the *TSLP* promoter activity, using an *in vitro* luciferase assay. The clone containing the -847T-82T haplotype showed greater transcriptional activity than those containing the -847C-82C haplotype when assayed under overexpression of AP-1 in the presence and absence of poly(I:C) (Figure 4D). Thus, AP-1 was shown to be involved in activation of the long-form *TSLP* promoter through the SNP -847C > T (rs3806933).

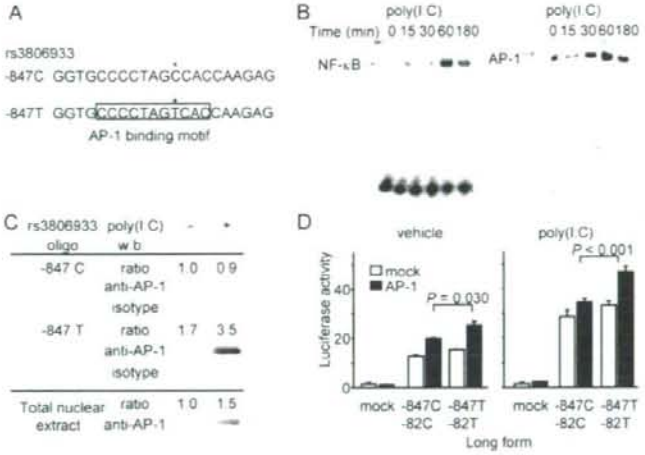

Figure 4. Differential transcription factors binding to regulatory SNP -847C > T (rs3806933). (*A*) The DNA sequences of transcription factor-binding motifs around SNPs in the *TSLP* long isoform. The positions of potential activating protein (AP)-1–binding sites are shown in the *open boxes*, and the *asterisks* represent the SNP. (*B*) NF-κB and AP-1 activation in NHBE stimulated with poly(I:C) *in vitro*. The *arrows* indicate the induced NF-κB (*left*) and AP-1 (*right*) complexes. (*C*) Binding affinity of transcription factors to oligonucleotides *in vitro*. Arbitrary densitometric units are shown above each band. Three independent experiments were performed with similar results. (*D*) AP-1–mediated promoter activity of the long-form *TSLP*. NHBE were transiently transfected with a *TSLP* long form promoter-driven luciferase reporter plasmid with expression vectors for AP-1 (*c-jun* and *c-fos*) or a mock vector. After 24 hours, NHBE were stimulated with or without poly(I:C) for 4 hours. Promoter activity was measured by luciferase assay.

DISCUSSION

The crucial role of the cytokine TSLP in the development of dermatitis and airway inflammation has been recognized. Of the two splice variants of the *TSLP* gene, only long-form *TSLP* was markedly induced by poly(I:C)/dsRNA stimulation in normal human airway epithelial cells and detected as TSLP protein with ELISA. Furthermore, we identified a functional SNP in the promoter region of the long-form *TSLP* that had an allele-specific effect on expression through varying affinity for the transcription factor AP-1.

Human *TSLP* is highly expressed by airway epithelial cells during allergic inflammation and potently activates immature $CD11c^+$ myeloid DCs (1). TSLP-activated DCs induce naïve $CD4^+$ T cell differentiation into Th2 cells that produce allergy-related cytokines (1–5, 8). TSLP induces human myeloid DCs to express OX40L, a member of the TNF family, and OX40L on TSLP-activated DCs triggers Th2 cell polarization, but OX40L loses the ability to polarize Th2 cells in the presence of IL-12 (23). Asthma is characterized by a Th2-type inflammation, and individuals with asthma are more susceptible to rhinovirus (RV) infections than normal individuals, having longer duration of lower respiratory tract symptoms when infected with RVs (24–26). A defective type 1 response to RVs in individuals with atopic asthma has been reported (27). When peripheral blood mononuclear cells were exposed to an RV and assessed for type 1 and type 2 cytokine production, IFN-γ and IL-12 concentrations were significantly higher in cells from normal individuals than in those from subjects with atopic asthma (27). RVs are RNA viruses that secrete a substantial level of dsRNA right after infection at the respiratory epithelium (11), and the -847T allele might cause overinduction of long-form *TSLP* transcripts via dsRNA stimulation of respiratory epithelial cells. Although little is known about what makes epithelial cells produce TSLP, the dominance of OX40L induced by TSLP over reduced IL-12 production in subjects with asthma may provide an explanation for Th2-dominant inflammation in asthma exacerbation. Overproduction of long-form *TSLP* caused by promoter polymorphisms in bronchial epithelial cells may lead to redundant Th2 responses and influence the host response to viral infection.

It is well accepted that the epithelium is an important therapeutic target for the treatment of asthma (15). Targeting TSLP itself is thought to be therapeutically efficacious as a new treatment for allergic disease (2, 28). Further studies to explore the roles of TSLP in respiratory viral infection and allergic inflammation would contribute to selecting patients most likely to respond.

Glucocorticosteroids are the most common anti-inflammatory drugs and are used as the first-line therapy for controlling asthma (29). These drugs bind to glucocorticoid receptors and inhibit the NF-κB– and AP-1–mediated transcription of various proinflammatory molecules through the glucocorticoid receptor–binding sites of their gene promoters (29, 30). Activation of NF-κB is critical for inflammation-induced expression of *TSLP* in airway epithelial cells, and an NF-κB–binding site was identified 3.7 kb upstream from the start of long-form *TSLP* transcription (17). In this study, we could not find any polymorphism around the NF-κB binding sequence. The promoter region of long-form *TSLP* containing -847T showed greater binding activity to AP-1 after dsRNA stimulation in human bronchial epithelial cells. We also found that the clone containing -847T showed greater transcriptional activity than those containing -847C when assayed under overexpression of AP-1. AP-1 is a prominent transcription factor in airway diseases and regulates the expression of multiple inflammatory proteins (30–32). These results suggest that the promoter *TSLP* SNP upregulates the microbe-induced production of TSLP by bronchial epithelial cells through enhancement of AP-1 binding to the promoter. AP-1 expression is enhanced in the asthmatic airway, and it is reduced after glucocorticoid therapy (32, 33). It is possible that the polymorphism of the *TSLP* gene-producing AP-1 site is related to the drug responsiveness.

In summary, we have demonstrated that the expression of long-form *TSLP*, which is a crucial cytokine for the induction of inflammatory Th2 responses, is highly induced by dsRNA in bronchial epithelial cells, as well as a functional promoter SNP that has an allele-specific effect on expression through altering affinity for the AP-1. These genetic factors of *TSLP* may influence the host response to viral infection, and knowledge of them will contribute to a better understanding of the pathogenesis of Th2-mediated respiratory diseases such as bronchial asthma.

Conflict of Interest Statement: M.T., Y.N., T.H., and M.H. applied for a patent regarding TSLP functional SNP in Japan. (Oct 31, 2006. No.2006-296561). S.F.Z. has stock in Amgen. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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平成19年度　気道過敏性試験の診療実態に関するアンケート調査報告

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1．はじめに

近年，喘息を含むアレルギー疾患の増加，さらに低年齢化が進んでいることが報告されている．我々の群馬県の小学生の検討でも，最近8年間の平均では，喘鳴の認められたことのある児は全体の22.4%，喘息と診断された児は10.8%であった．小児の喘息を巡る日常診療で問題となるのは，喘息と診断された小児だけでなく，このような喘鳴のみと答える小児，さらには，喘息のアウトグローを訴える小児への対応である．この中には，詳細な問診によれば運動時や感染症罹患時に発作を起こしていることが確認される症例も少なくないため，喘息の診断や重症度を評価するための客観的な指標は重要である．

この点，気道過敏性の測定は望ましいものと思われる．今回の調査結果から，今日の小児アレルギー学会会員における気道過敏性の試験施行の現状について検討したため，報告する．

2．対象ならびに方法

日本小児アレルギー学会社会保険委員会として，本邦における小児の気道過敏性試験に関連する診療実態を把握し，保険診療報酬を適正化する資料を得るため，本委員会で作成した調査用紙を用いて平成19年11月にアンケート調査を施行した．

対象は日本小児アレルギー学会会員2820名であるが，このアンケートの設問に回答のあったのは816施設で，その中で気道過敏性試験を行っている施設は79施設（9.7%）であった．

3．結　果

回答のあった79施設の結果を以下に述べる．気道過敏性試験を開始する年齢層については，乳児8（10.1%），幼児5（6.3%），小学生61（77.2%），中学生2（2.5%），その他2（2.5%）であった．問9に回答のあった「乳幼児に気道過敏性試験を行っている施設」は19施設で，メサコリンで経皮的酸素飽和度測定5（26.3%），ヒスタミンで経皮的酸素飽和度測定2（10.5%），メサコリンで経皮的酸素分圧測定3（15.8%），ヒスタミンで経皮的酸素分圧測定7（36.8%），その他2（10.5%）であった（図1）[n=19]．月平均回数は，5件未満が11（57.9%），5-10件が7（36.8%），11-20件が1（5.3%），21件以上は0であった（図2），検査にかかる時間では，30分は0，1時間は17（89.5%），2時間は2（10.5%），それ以上は0であった（図3）．乳幼児の検査中に呼吸困難などの緊急対応を必要とした症例の経験の有無について，42施設が回答しているが，有りが18（42.9%），無しが24（57.1%）であった（図4）．

小学生以降の年齢で気道過敏性を測定している施設は79施設で，アセチルコリンで標準法21（26.5%），メサコリンで標準法30（38.0%），ヒスタミンで標準法5（6.3%），アストグラフ法12（15.2%），複数の方法にて11（13.9%）であった（図1）[n=79]．月平均回数は，5件未満が46（58.2%），5-10件が20（25.3%），11-20件が4（5.1%），21件以上は1（1.3%），無回答8（10.1%）であった（図2）．検査にかかる時間では，30分は15（20.0%），1時間は（33（41.8%），2時間は19（24.1%），それ以上は3（3.8%），無回答は9（11.4%）であった（図3）．検査中に呼吸困難などの緊急対応を必要とした症例の経験の有無については72施設が回答しているが，有りが35（48.6%），無しが38（52.4%）であった（図4）．