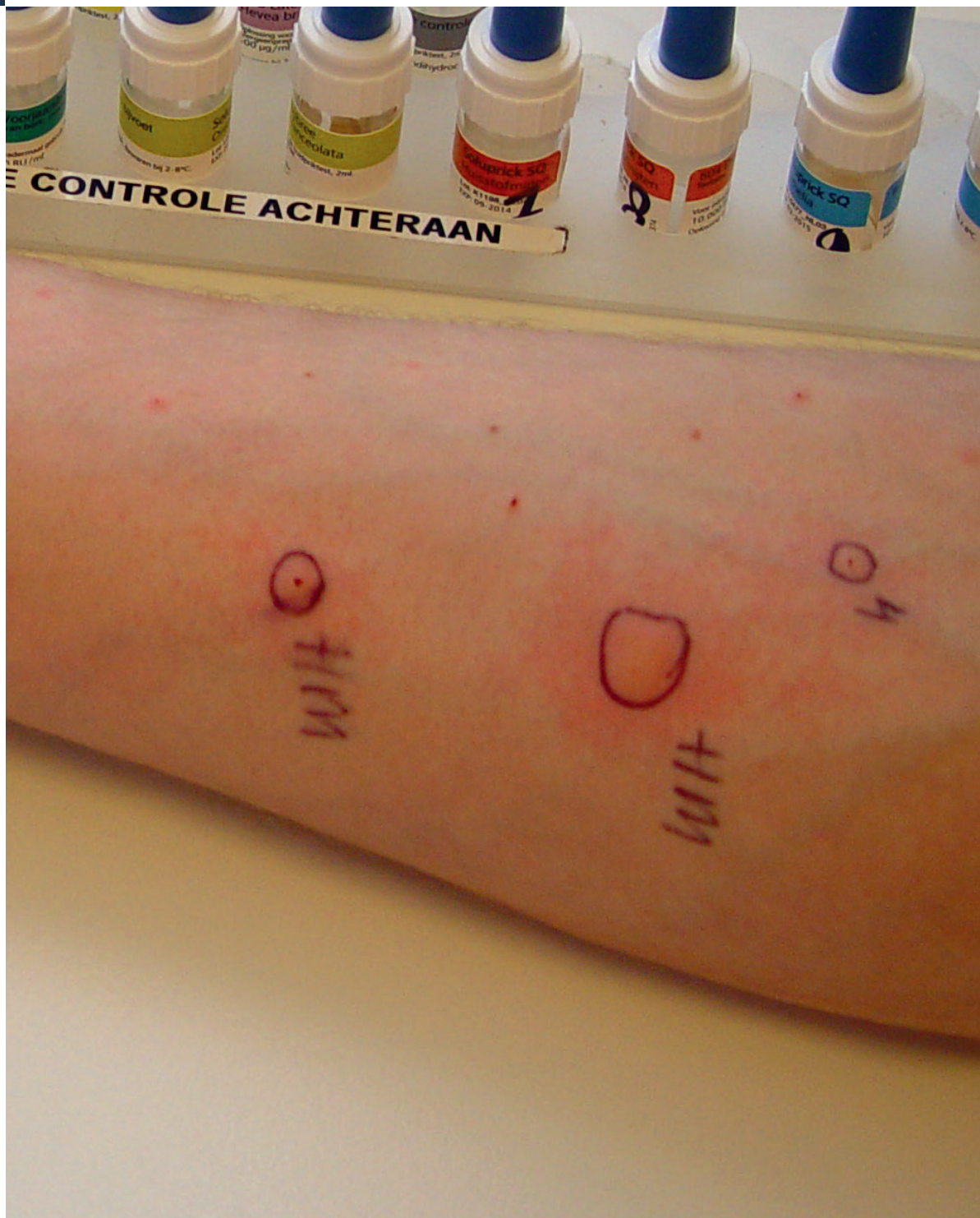
Recombinant Allergens

Working safely with recombinant allergenic biologicals



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Working safely with recombinant allergenic biologicals

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Disclaimer

Foreword

Allergy is a growing concern in modern society and it is therefore no surprise that scientists more and more exploit genetic modification to produce and modify allergens for scientific, diagnostic and therapeutic purposes. Furthermore, allergens may also be introduced unknowingly by genetic modification.

COGEM and the Dutch GMO office are confronted with increased activities in this field. While the Dutch law and regulations explicitly address the safe production and use of genetically modified organisms (GMOs) expressing harmful substances, they do not pay specific attention to the expression of allergens. The question for COGEM and the GMO office was which measures are required to protect the safety of man and environment when working with GMOs expressing allergens.

In this report, Dr. Rüdelsheim and Dr. Smeets present an overview of the perspectives enabled by molecular cloning of allergens, the risks of exposure of people, in particular laboratory workers, to high doses of allergens, and the protective and containment measures that are available to limit allergen exposure.

Steps in the risk assessment and control of GMOs expressing known or potential allergens include an assessment of the allergenicity of the expressed protein, similar to an assessment of its toxicity, and an assessment of the likelihood that the hazard will actually be realised. This depends on the nature, the level, and location of expression of the toxic or allergic protein, and the type of activities. As for now, the same approach is used in the risk assessment for toxins and allergens. In contrast to toxins, however, an important feature of working with allergens is that a safe threshold cannot be determined. Even very small quantities of allergens may already be harmful to certain individuals, in particular genetically predisposed individuals. This makes allergens a special type of hazardous substance that may require stringent containment measures, for example to limit the risk of exposure by aerosols or skin contact.

To help the risk assessment, the authors propose a classification of proteins with regard to their allergenicity. Evidently, also the spread of the GMO beyond containment should be prevented. The authors emphasize that when the GMO itself is inactivated or no longer present, for example after purification of the allergen, the safety of the laboratory workers is no longer guaranteed by GMO legislation but by the employment protection legislation. The authors present several case studies to illustrate the risk assessment procedure and proposed containment management measures when working with specific allergens in specific activities.

In conclusion, the report provides a valuable overview of the considerations that research workers, the COGEM and the GMO office may use in their risk assessment and risk management procedures when working with GMOs expressing allergic proteins. The Advisory Committee trusts that the report will be a valuable instrument to help working safely with GMO-produced allergens.

Tjeerd G. Kimman
Chairman of the Advisory Committee

Summary

Over the last decades, the incidence of allergic disease has been almost continuously increasing. The growing awareness concerning allergic disease has led to a cautious risk classification of activities in contained use (CU) with genetically modified organism (GMOs) that are intended to produce allergens. The increasing number of CU applications with recombinant allergens requires a review of the scientific knowledge and current practices as a basis to evaluate if this cautious approach is still adequate for all cases.

The human immune system is a complex defence mechanism able to protect from pathogens and foreign substances. Allergy is a heterogeneous disorder of the immune system that despite increasing knowledge is not completely understood. The risk factors for allergic disease are a person's predisposition, the exposure to allergens and the environment.

Allergens may be deliberately expressed in transgenic research or introduced unknowingly. In both cases the allergenic potential of the targeted GMOs needs to be evaluated. Combined with an exposure analysis the risks related to the specific activity can be derived. This risk assessment is the basis for reviewing risk management measures to protect workers from sensitisation and/or allergy development. The focus of this report is about well-known protein allergens that may be inhaled or come into contact with the skin and mucosa.

Allergens are most challenging amongst hazardous substances. There are models for predicting allergenicity of low molecular weight (skin) contact allergens, but no established models for respiratory and food allergens. Threshold values below which adverse effects do not occur are hard to determine because of individual predisposition, the variability between and even within persons, non-linear dose-response relationships, etc. Further complicating factors are the large diversity of allergens, cross-reactivity and contribution of environmental influences. Furthermore, the fact that the reaction involves typically two phases, sensitisation, which may progress unnoticed, and elicitation, adds additional complexity to the evaluation.

The development of molecular cloning techniques enables production of recombinant allergens in a standardised and well characterised way for basic research, diagnostic and therapeutic purposes (*e.g.* allergen immunotherapy). Hence, the interest to perform research on and produce allergens based on GMOs in contained facilities. However, such activities may also create unprecedented situations where people get exposed in new settings to high doses of an allergen. The growing awareness of the increased prevalence of allergic disease inspires a cautious approach to limit allergen exposure. While this would already be expected by workers protection legislation, this is further enforced for GMOs for which a risk assessment preceding any activity is a legal requirement.

Within this context, the aim of this study is to review information on allergens expressed by GMOs, and the associated risks for people working with these GMOs in containment facilities. Based on case studies, a proposal is made to classify GMOs in relation to their ability to cause allergic reactions and to determine appropriate protective/containment measures related to different activities.

As a first component in the risk assessment, it is proposed that the classification of proteins may include elements such as prevalence of the corresponding IgE in the allergic population, occurrence in the environment, severity of disease, and cross-reactivity potential. On this basis, four groups of proteins can be distinguished, namely known allergens (scientifically established evidence), probably allergenic proteins (some indications available), proteins for which allergenicity cannot be excluded (single cases, ill-defined studies), and proteins with no indication of allergenicity. Based on this classification, proteins in group 1 and 2 would be considered hazardous substances, whereas group 4

proteins would not. For group 3 proteins additional evaluation or cautionary preventive measures would be warranted.

The second element in the risk assessment is the likelihood of the hazard to be realised. This is defined by the potential for exposure and the health status of the laboratory worker. Exposure is determined by the type of handlings as well as by the expression of the protein, the level of expression and location (*e.g.* intracellular, in the cell wall, or excreted). This may necessitate dividing activities into several steps, each resulting in a corresponding risk classification.

In general the amounts one might be exposed to in CU conditions are rather low. However, a safe threshold for working with allergens cannot be determined. Even in large-scale production facilities where substantial quantities may be produced, exposure will be limited, as the culture of a GMO already requires stringent containment measures. It is noticed that extractions from natural allergen sources, or the purification steps after a recombinant allergen has been produced, may pose a much higher risk. Such activities not involving viable biological material are not governed by GMO regulations, but worker's safety remains guaranteed by workers protection legislation.

The most prominent exposure routes are inhalation of aerosols and contact via skin or mucosa. While the basic set of good laboratory practices for dealing with GMOs at biosafety level (BSL) I will already provide some protection, additional protective measures may include the use of a microbiological safety cabinet (MSC) class II, specific respiratory protection and gloves. To a great extent this can be achieved by requiring a BSL II containment. For specific cases - to be determined case-by-case -, *e.g.* a group 3 protein, a BSL I with additional precautions may provide a suitable alternative. This option may offer a bridge to other activities (*e.g.* establishing gene banks, working with organisms with endogenous allergens) that are not subject to high containment requirements.

Since the hazard potential is predominantly determined by the worker, making employees aware of the potential risk is important. Most adults know which sources they are allergic to, as sensitisation most often occurs in childhood. Extra precautions or refraining from 'risky' activities may substantially reduce the possibility of an adverse reaction. In addition medical surveillance and presence of an emergency kit may be useful.

Although persons with atopic disease (allergic asthma, rhinitis, eczema) may be made aware of the risks and may be additionally protected, others need to be protected as well. While presently activities with allergen producing GMOs are categorised to a certain biosafety level as a whole, a subdivision may provide an opportunity to classify certain handlings to a lower level allowing for a more convenient working environment.

Samenvatting

Gedurende de laatste decennia neemt de prevalentie van allergieën bijna continue toe. Het groeiende besef over de ernst van allergieën heeft geleid tot een voorzichtige risicobeoordeling voor het ingeperkt gebruik van GGOs die bedoeld zijn om allergenen te produceren. Het toenemend aantal vergunningaanvragen voor ingeperkt gebruik van recombinante allergenen vraagt om een overzicht van de huidige stand van de wetenschap en de gebruikelijke praktijk als basis van een evaluatie of deze voorzichtige benadering nog steeds geschikt is voor alle gevallen.

Het menselijke immuunsysteem is een complex afweersysteem dat beschermt tegen ziekteverwekkers en lichaamsvreemde stoffen. Allergieën zijn een heterogene groep van aandoeningen van het immuunsysteem die ondanks de toegenomen kennis nog niet volledig worden begrepen. De risicofactoren om een allergie op te lopen omvatten iemands genetische constitutie, de mate van blootstelling en het milieu.

Allergenen kunnen doelbewust tot expressie worden gebracht in recombinant DNA-onderzoek, maar ook onbewust. In beide gevallen moet de mogelijke allergeniciteit van de bedoelde GGO's worden onderzocht. Het eigenlijke risico kan dan ingeschat worden rekening houdend met de kans op blootstelling. Deze risicoanalyse is de basis voor verdere risicobeheersmaatregelen om medewerkers te beschermen tegen sensibilisatie en/of het uiteindelijk ontstaan van een allergie. In dit rapport ligt de nadruk vooral op werken met bekende eiwitallergenen waarmee medewerkers mogelijk in aanraking komen via inademing of huid- en slijmvliescontact.

Allergenen zijn een bijzondere en complexe categorie binnen de schadelijke stoffen. Er bestaan wel modellen om de allergeniciteit van contactallergenen met een laag molecuulgewicht te voorspellen, maar geen die voldoen voor inhalatie- of voedselallergenen. Het is zeer moeilijk om drempelwaarden vast te stellen waaronder geen schadelijke effecten optreden. Dit komt onder meer door de individuele aanleg, de verschillen in gevoeligheid tussen mensen en zelfs bij één individu, het niet-lineaire verband tussen dosis en respons, enz. De grote diversiteit aan allergenen is een bijkomende complicerende factor. Verdere complicerende factoren zijn het voorkomen van kruisreactiviteit en de invloed van milieufactoren. Een laatste moeilijkheid is het feit dat de aandoening twee fasen kent: een sensibilisatiefase, die vaak onopgemerkt verloopt, en de eigenlijke allergische reactie.

De ontwikkeling van moleculaire kloneringstechnieken heeft het mogelijk gemaakt dat recombinante allergenen op een gestandaardiseerde en goed gekarakteriseerde wijze kunnen worden aangemaakt voor fundamenteel onderzoek, diagnostiek en therapie (bv. immunotherapie). Vandaar ook de interesse voor onderzoek aan en productie van allergenen met recombinante technieken in ingeperkte omstandigheden. Tegen deze achtergrond kunnen zich echter onbekende situaties voordoen waarbij medewerkers worden blootgesteld aan hoge doses van een bepaald allergeen. Het groeiend bewustzijn van het toegenomen aantal allergieën maant tot een voorzichtige aanpak om blootstelling te beperken. Dit wordt al opgelegd door de wet op bescherming van de werknemer, maar wordt verder vereist in de voorafgaandelijke milieurisicobeoordeling voor werken met GGO's.

In deze context tracht deze studie een overzicht te geven van de kennis die bestaat voor GGO's die allergenen tot expressie brengen en de bijbehorende risico's voor mensen bij ingeperkt gebruik. Er wordt een voorstel gedaan om GGO's in te delen volgens hun vermogen om allergische reacties te veroorzaken en om gepaste beschermende en inperkende maatregelen vast te stellen bij de diverse werkzaamheden.

Als eerste component in de risicoanalyse wordt voorgesteld om bij de classificatie van eiwitten rekening te houden met elementen zoals het voorkomen van het overeenkomstige IgE in de allergische populatie, het voorkomen in het milieu, de ernst van de effecten en het vermogen tot

kruisreactiviteit. Op deze basis kunnen vier groepen onderscheiden worden, namelijk bekende allergenen (wetenschappelijk bewezen), mogelijk allergene eiwitten (indicaties zijn aanwezig), eiwitten waarvoor niet kan worden uitgesloten dat ze allergeen zijn (sporadische gevallen, dubbelzinnige studies en ontoereikende testmethoden) en eiwitten zonder indicatie van allergeniciteit. Volgens deze indeling zullen eiwitten uit groep 1 en 2 als schadelijke stoffen worden beschouwd, en die uit groep 4 niet. Voor groep 3 eiwitten worden bijkomend onderzoek of zorgvuldig uitgekozen voorzorgsmaatregelen nodig geacht.

Het tweede element in de risicobeoordeling is de kans dat het schadelijk effect zich voordoet. Dit wordt bepaald door de kans op blootstelling en de gezondheidstoestand van de medewerker. Blootstelling wordt op zijn beurt bepaald door het type werkzaamheden, het al dan niet tot expressie komen van het gen, het expressieniveau en –locatie (intracellulair, in de celwand of extracellulair). Deze overwegingen kunnen leiden tot het opsplitsen van een activiteit in verschillende onderdelen, elk met hun overeenkomstig risico-indeling.

In het algemeen zijn de hoeveelheden waaraan men kan worden blootgesteld bij ingeperkt gebruik eerder laag. Toch kan een veilige ondergrens niet worden vastgesteld. Zelfs bij grootschalige installaties waar relatief grote hoeveelheden worden geproduceerd, is blootstelling beperkt omdat de kweek van een GGO al strenge inperking vereist. Er wordt opgemerkt dat het extraheren van allergenen uit natuurlijke bronnen of het opzuiveren van een recombinant allergeen een veel groter risico kan inhouden. Deze activiteiten die niet gaan over levend biologisch materiaal worden niet gedekt door de GGO-regelgeving. De veiligheid van de werknemers blijft echter gegarandeerd door toepassing van de arbeidsomstandighedenwet.

De meest voorkomende mogelijkheden tot blootstelling zijn de inhalatie van aerosolen en contact met de huid of slijmvliezen. Hoewel goede laboratoriumpraktijken bij handelingen met GGO's op inschalingsniveau 1 al een zekere bescherming bieden, kunnen bijkomende beschermende maatregelen worden geadviseerd zoals het gebruik van een klasse II microbiologisch veiligheidskabinet, het dragen van ademhalingsbescherming en handschoenen. Een inschalingsniveau 2 voorziet deze mogelijkheden al ten dele. Voor specifieke gevallen –geval-per-geval te bepalen-, bv. voor groep 3 eiwitten, kan een inschalingsniveau 1 met bijkomende maatregelen een geschikt alternatief bieden. Deze optie biedt ook mogelijkheden voor andere activiteiten (bv. het aanleggen van een genenbank, handelingen met organismen met endogene allergenen) die geen hoge inperkingsmaatregelen vereisen.

Daar het mogelijke gevaar vooral afhangt van de medewerker, is het van belang het personeel hiervan bewust te maken. De meeste volwassenen weten voor welke stoffen ze allergisch zijn, omdat sensibilisatie meestal al in de jeugd is opgetreden. Extra voorzorgsmaatregelen of afzien van risicovolle handelingen kunnen al voldoende zijn om de kans op een schadelijk effect aanzienlijk te reduceren. Daarbovenop kunnen medisch toezicht en de aanwezigheid van een eerstehulpset nuttig zijn.

Hoewel personen met een atopie (allergische astma, rhinitis, eczema) zich bewust zijn van de risico's en extra beschermd moeten worden, moeten anderen evenzeer gevrijwaard blijven van ongewenste effecten. Terwijl tot nog toe handelingen met allergenenproducerende GGO's in hun geheel ingeschaald worden op een bepaald veiligheidsniveau, kan een verdere opsplitsing van de activiteiten in deelactiviteiten de mogelijkheid bieden bepaalde handelingen op een lager niveau in minder belastende werkomstandigheden te laten uitvoeren.

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Abbreviations

APC	Antigen-Presenting Cell
BSL	Biosafety Level
COGEM	Commissie Genetische Modificatie
CU	Contained Use
EAACI	European Academy of Allergology and Clinical Immunology
EFSA	European Food Safety Association
ELISA	Enzyme Linked Immunosorbent Assay
ERA	Environmental Risk Assessment
FAO	Food and Agriculture Organization of the United Nations
FcεRI	high-affinity IgE receptor
FcεRII	low-affinity IgE receptor
GG	Genetisch Gemodificeerd
GGO	Genetisch Gemodificeerd Organisme
GM	Genetically Modified
GMM	Genetically Modified Micro-organism
GMO	Genetically Modified Organism
GMT	Good Microbiological Techniques
IFN-γ	Interferon-gamma
IG	Ingeperkt Gebruik
Ig	Immunoglobulin
IL	Interleukin
IVC	Individually Ventilated Cage
MHC	Major Histocompatibility Complex
MSC	Microbiological Safety Cabinet
NK cells	Natural Killer cells
OECD	Organisation for Economic Co-operation and Development
PAPR	Powered Air Purified Respirator
PG	pathogenicity class
PPE	Personal Protective Equipment
RA	Risk Assessment
SIT	Allergen-Specific Immunotherapy
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-beta
WAO	World Allergy Organization
WHO	World Health Organization

Glossary

Adjuvant is a substance that, when co-administered with an antigen, increases the immune response to that antigen. Also, any substance that acts to accelerate, prolong, or enhance antigen-specific immune responses when used in combination with specific vaccine antigens.

Allergens are antigens which cause allergy. Most allergens reacting with IgE and IgG antibody are proteins, often with carbohydrate side chains, but in certain circumstances specific pure carbohydrates have also been proposed to be allergens. Skin contact allergens causing eczema are usually low molecular substances (*e.g.* nickel).

Allergenicity is the potential of a substance to cause an allergic reaction or allergy.

Allergy is a hypersensitivity reaction initiated by immunological mechanisms. Allergy can be antibody- or cell-mediated. It is manifesting in objectively reproducible symptoms.

Anaphylaxis is an acute severe, life-threatening, generalised or systemic hypersensitivity reaction. When an immunologic mechanism is involved this is termed an allergic anaphylaxis.

Antibody: see **immunoglobulin**

Antigen is the substance that binds specifically to the respective antibody and/or to immune competent cells.

Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils and T lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or in the early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneously or with treatment. This inflammation also causes an associated increase in airway responsiveness to a variety of stimuli.
Allergic asthma is the basic term for asthma mediated by immunological mechanisms.

Atopy is a genetic predisposition to development of specific IgE antibodies against common allergens. Atopy is a personal and/or familial tendency, usually in childhood or adolescence, to become sensitised and produce IgE antibodies in response to ordinary exposure to allergens, usually proteins.

Cross-reactivity is the phenomenon where a substance is able to bind with an antibody that was raised to a different antigen (immunologic recognition). T cell cross-reactivity requires similarity in short peptides, known as T cell epitopes. B cell cross-reactivity requires similarity in 3D structure, not only in the strictly architectural sense, but also in the physicochemical properties such as similar charge distribution, hydrogen-bonding potential and hydrophobicity patterns.

Elicitation is an allergic reaction following an encounter with an allergen one is previously sensitised to.

Epitope is a molecular region on the surface of an antigen capable of eliciting an immune response and for binding of the specific antibody produced by such a response.

Hapten is a specific non-protein substance, which does not itself elicit antibody formation but does elicit the immune response when coupled with a carrier protein. It is a small molecule that stimulates the production of antibody molecules and/or immune competent cells only when conjugated to a larger molecule, called a carrier molecule. The hapten is covalently bound to the carrier molecule, most often

a protein. The hapten-carrier complex stimulates the production of antibodies and/or immune competent cells, which the unbound hapten cannot do, and becomes immunogenic (capable of eliciting an immune response).

Haptenisation is the combining of an antigenic compound with a carrier protein molecule, capable of stimulating an immune response.

Hypersensitivity causes objectively reproducible symptoms or signs, initiated by exposure to a defined stimulus that is tolerated by normal subjects. It may include sensitivity to allergens and intolerance.

- Immediate-type hypersensitivity is associated with allergen-specific antibodies (IgE) in serum;
- Delayed-type hypersensitivity is associated with allergen-specific effector T cells.

Immunogenicity is the capability to elicit an immune response.

Immunoglobulin is a protein with antibody activity produced by plasma cells of the immune system. These proteins are comprised of heavy and light chains with constant and variable regions that determine antigen specificity. There are 5 known human immunoglobulin isotypes.

Lymphocyte is an immune cell derived from a hematopoietic stem cell through a process called lymphopoiesis and thymopoiesis. The two main types of lymphocytes are B-lymphocytes, which develop into plasma cells that produce antibodies, and T lymphocytes, which serve as the central type of cell in the immune response. T cells comprise several different subsets with different functions, and provide help to B cells.

Sensitisation is the induction of a specialised immunological memory in an individual by exposure to an allergen. The immune system responds with a specific cellular and/or humoral response (IgE, IgG, etc.) to the allergen in question, a process that by itself does not cause any symptoms.
Also, sensitisation may be defined as the process by which a person becomes, over time, increasingly allergic to a substance (sensitizer) through repeated exposure to that substance.

- Co-sensitisation: independent sensitisation to two or more different allergen sources.
- Cross-sensitisation: sensitisation to cross-reactive allergens (sensitisation to one molecule may evoke symptoms when in contact to another structurally similar molecule).

Threshold is the lower exposure limit below which an allergen will not cause any symptoms.

Tolerance is unresponsiveness, or a non-inflammatory response to an antigen that is induced by immunologic mechanisms that develop because of previous exposure to that antigen.

1 Introduction

Preceding any activity with GMOs a risk assessment must be conducted. In addition to the characteristics of the recipient organisms, potential adverse effects of the proteins encoded by the newly inserted sequences are key-elements of this assessment. If a gene product is found to be (potentially) harmful, protection measures are required to protect people and the environment that may be exposed by the activity.

Next to toxins, allergens are considered a class of harmful gene products that require specific attention. Yet, in contrast to toxins, the approach for dealing with allergens is less developed. In the Western World an increase in the prevalence of allergic disease has been noticed in the past few decades (WAO, 2011). The complex relation between illness and exposure to allergens has further inspired a cautious classification of CU of GMOs modified to express so-called strong allergens as high-risk level activities (*e.g.* IG 02-063/02, IG 03-188/03 and IG 12-109/00).

Natural allergen extracts consisting of mixtures of allergenic and non-allergenic components of varying quality have been and are still being used to diagnose allergies. To preserve the allergenic potency a minimum of processing is performed. With the development of molecular cloning techniques it becomes possible to produce and modify recombinant allergens in a standardised and well characterised way for basic research, diagnostic and therapeutic purposes (*e.g.* allergen immunotherapy). Hence, the interest to perform research on and produce recombinant allergens in contained facilities.

While this is relevant for GMOs intended to produce proteins with known allergenic potential, other proteins may be introduced that can trigger an allergenic reaction and that have not been identified before as such. Each user therefore will need tools to judge *a priori* the allergenic potential of the targeted proteins.

Combined with an exposure analysis this will lead to a better understanding of the risks related to the specific activity and can be the basis for designing appropriate risk management measures to protect workers from sensitisation and/or allergy development.

Aim and scope

The information presented in this report aims to support the risk assessment for work with GMOs expressing allergens. Risks are identified and a scheme to classify GMO activities with (potential) allergens is proposed. Arguments for risk management are given reviewing commonly encountered situations of exposure and related suitable containment measures.

This report focuses on potential occupational health impacts when dealing with GMOs in CU that may cause allergic effects. These are typically research activities, possibly involving animals, but also small and large-scale productions of GMOs can be envisaged. Furthermore, it takes into consideration both allergies that may be induced by newly expressed proteins and those that may be induced by up-regulated endogenous allergens. Given the focus on CU, the main exposure routes are expected to be via skin contact and inhalation (ingestion as prominent in food use is excluded at this stage).

Approach

This report provides an introduction to the latest insights on allergy development when working with potential allergens. Therefore, a brief overview of the state of knowledge of the healthy immune system is presented, followed by information on allergic reactions. A more detailed review is given in Annex I. In assessing risks, the characteristics of the GMO in relation to allergy are discussed and they are reviewed in function of the feasibility to predict the allergenic potential. The risk assessment is further documented by identifying exposure scenarios in CU conditions relevant for allergies. Also, the

feasibility of a classification system for allergenic proteins is discussed. Finally, an overview is presented of preventive measures in line with the overall conditions that prevail for different confinement levels. The risk assessment approach is further illustrated by selected case studies.

2 Current state of knowledge

2.1 The immune system

The immune system is the mechanism that defends the body from invading organisms and molecules. It is a layered defence system with a first **physical barrier** (skin and mucosa), and a second barrier called the **innate immune system** that is a non-specific immune system. The third layer of protection is the **adaptive immune system** that is activated by the innate response, and is a pathogen and antigen specific response.

The innate response acts immediately (within hours) and slows down the spread of the infection. Its function is to recruit immune cells to sites of infection via chemical mediators, the cytokines. It removes dead cells and antibody complexes. It triggers the adaptive immune response through an antigen presentation process. The cellular components are leukocytes including natural killer cells, mast cells, eosinophils, basophils, and the phagocytic cells including macrophages, neutrophils and dendritic cells.

The adaptive response is antigen-specific and may last for days to weeks. The receptor cells that recognise the antigen are lymphocytes (B cells and T cells). In B cells the cell-surface receptors for pathogens are immunoglobulins (Ig), whereas for T cells one speaks of T cell receptors (TCR). T cells recognise antigens only after they have been processed and presented in combination with a receptor called a major histocompatibility complex (MHC) molecule. Recognition by B cells happens without processing of the antigen.

The first time that an adaptive immune response to a certain organism or substance is induced, is referred to as the primary immune response. Any following reaction to the same organism is called a secondary immune response.
After exposure to an antigen, B cells differentiate into plasma cells whose primary function is the production of antibodies. They are presented on the surface of the B cell and when bound to the antigen are taken up by the B cell, processed by proteolysis into antigenic peptides and displayed on the surface bound to MHC class II molecules. This complex attracts the matching T helper cell that releases lymphokines (cytokines) and activates the B cell to divide. The B cell offspring (plasma cells) secrete millions of copies of the antibody that recognises this antigen. These antibodies circulate in blood plasma and lymph, bind to pathogens expressing the same antigen and mark them for destruction.

After contact with the antigen, naïve helper T cells (T_H0) differentiate into inflammatory T_H1 cells, helper T_H2 cells or pathogenic T_H17 cells, depending upon the cytokines in the environment. T_H1 cells activate macrophages and participate in the generation of natural killer cells. T_H2 cells help to activate B cells, resulting in antibody production. A complex regulating mechanism directs the immune response in function of the type of threat. Depending on the type of cytokines that T_H2 cells produce the B cells will switch to different classes of antibody production adapting to the different types of pathogens/substances.

When B cells and T cells are activated and replicate, some of their offspring become long-lived memory cells. Memory cells will induce the secondary immune response upon the next contact with the invader.

In order not to attack harmless antigens (food, commensal micro-organisms, environmental antigens, the body's own antigens) regulatory T cells (Treg) are found to be key in regulatory mechanisms for tolerance to these antigens, including allergens. However, knowledge of the molecular mechanisms in

Treg cell–mediated immune regulation is still limited. Two cytokines are very important in regulatory T cell function: transforming growth factor (TGF-β) and interleukin-10 (IL-10). Both play a role in inhibiting inflammatory pathologies.

T_H1, T_H2, T_H17 and Treg cells regulate each other through positive and negative feedback mechanisms.

2.2 Allergy and hypersensitivity

Hypersensitivity can be described as a peculiar or excessive susceptibility to a specific factor, that occurs in a minority of the population. Hypersensitivity may encompass both immune based and non-immune based disorders (*e.g.* lactose intolerance).

Allergy is, strictly speaking, different from hypersensitivity in that it requires specific recognition by the immune system. The body reacts to normally harmless substances in the environment. It is an overreaction of the adaptive immune system.

Not everyone reacts to an antigen exposure with an allergic response. Some people suffer from disease where most others do not show any symptom. The mechanism of allergic responses is heterogenous. The most prevalent form of allergy is the so-called type I form (Gell & Coombs, 1963) Type I allergy is believed to result from a disturbed balance between the different T cells. When T_H2 cells predominate over T_H1 cells, this results in an allergic reaction. The mechanism behind the shift from T_H1 to T_H2 is not known. However, dominant regulatory mechanisms with Treg cells in a central role may be essential (Agua-Doce & Graca, 2012; Hawrylowicz & O'Garra, 2005; Josefowicz *et al.*, 2012).

2.2.1 Mechanisms of allergy development

Two phases are recognised in allergy: sensitisation and elicitation.

Sensitisation is the induction of a specialised immunological memory in an individual by exposure to an allergen. The process that by itself does not cause any symptoms.
When an allergen is encountered for the first time, it is taken up, processed and presented as a peptide-MHC complex to the T_H2 cell. Upon recognition, cytokines (IL-4, IL-13) and CD40 ligand are released stimulating B cells. In type I allergy B cells start the production of IgEs instead of producing IgA or IgG. The IgE then binds to a high-affinity receptor FcεRI on the surface of mast cells and basophils.

Only after the second and subsequent exposure an allergic reaction is induced (elicitation or allergy phase).
The allergen binds to the corresponding IgE coated on the surface of the sensitised mast cells or basophils. Cross-linking more than one IgE with the same antigen activates the sensitised cell triggering a complex intracellular signalling process resulting in the secretion of biologically active molecules. Sensitised cells release histamine and other inflammatory chemical mediators (cytokines, interleukins, leukotrienes, and prostaglandins) from their granules into the surrounding tissue causing several systemic effects, such as vasodilation (widening of blood vessels), mucous secretion, nerve stimulation and smooth muscle contraction (*e.g.* in the longs obstructing airflow resulting in wheezing) (early-phase reactions). This leads to erythema (reddening) of the skin, rhinorrhea (runny nose), dyspnea (shortness of breath), itchiness, urticarial (hives), vomiting, diarrhoea, and anaphylaxis. Six to 24 hours later this is followed by a persistent oedema (swelling from fluid accumulation) and a

leukocytic influx (late-phase reaction). In the lungs it is believed to play a major role in the genesis of persistent allergic symptoms.

Although initially the focus of understanding was on an imbalance between T_H1 and T_H2 response, it is now clear that the causes should be searched more upstream, concentrating on genes involved in the control of inflammation and innate immune responses. While TGF-β and IL-10 both protect from allergic diseases, allergic individuals express lower levels of IL-10 that normally inhibits mast cell degranulation. In patients with allergies the function of Treg cells may be reduced or altered compared with normal healthy individuals.

Depending on the individual, the allergen, and the mode of introduction, the symptoms can be system-wide (classical anaphylaxis), or localised to particular body systems: *e.g.* asthma to the respiratory system, eczema to the dermis. Also, total IgE levels are influenced by age, genetic makeup and race, immune status, environmental factors (*e.g.* season of the year for hay fever), and disease process. Moreover, the threshold levels for an allergen to cause a reaction may be different for different persons. Even more, there is no linear relationship between the amount of an allergic protein and the severity of the disease.

Next to type I allergy other allergic responses may occur (type II to IV) that are less acute in onset, involve different immune mechanisms and usually require a higher degree of exposure to elicit symptoms (see Annex).

2.2.2 Influencing factors

2.2.2.1 Atopy

Atopy is a predisposition towards developing certain allergic reactions and is partly genetically determined next to environmental factors. It usually starts in childhood or adolescence, becoming sensitised and producing IgE antibodies in response to ordinary exposure of allergens, usually proteins. Atopic patients often suffer from more than one kind of allergic reaction, such as eczema (atopic dermatitis), allergic rhinitis (*e.g.* hay fever), allergic conjunctivitis, or allergic asthma.

2.2.2.2 Haptenisation

While an IgE-mediated immune response is provoked by large molecules, such as proteins, small molecules (<1000 dalton) may elicit such an immune response as well, but only when attached to a large carrier such as a protein. These molecules are termed haptens. The response is T cell-mediated.

2.2.2.3 Adjuvants and environmental factors

Adjuvants are defined as non-immunogenic substances that may stimulate the immune system, without being an antigen itself, *i.e.* substances that, when co-administered with a protein increases its immunogenicity and therefore might increase as well its allergenicity.
Substances may as well facilitate allergic reaction by irritation: chemicals may damage airway epithelia cells, the first barrier, resulting in an increased actual exposure to the allergen.

Pollution, pathogens, a person's lifestyle (dietary, occupational factors) may influence the prevalence of allergies.

2.2.3 Characteristics of allergens

The properties of an antigen that determine its allergenicity are its epitopes. Epitopes that interact with B cells may be localised on distant parts of the amino acid sequence, but come together in the tertiary structure. T cells only recognise short peptides as presented by the immune cells. Therefore, epitopes recognised by B cells are essentially conformational in nature, whereas epitopes recognised by T cells are linear.
Allergens further may have two properties: the property to sensitise, that is to induce the immune system to produce IgE specific to the allergen, and the property to elicit an allergic reaction. Some proteins, however, are known to elicit '*allergic*' symptoms but do not usually sensitise (Aalberse, 2000).

2.2.4 Cross-reactivity

Sometimes an antigen is able to bind with an antibody that was raised to a different antigen. This is called cross-reactivity. The definition of cross-reactivity is based on immunologic recognition: two allergens are cross-reactive if there is a single antibody (or T cell receptor) that reacts with both. (Aalberse *et al.*, 2001).
Cross-reactivity exists between antigens from a variety of distinct sources. The underlying cause may be shared epitopes on multivalent antigens or a conformational similarity of epitopes. In the first case the binding affinity is the same as for the original antigen, in the second case antibodies would bind with lesser affinity (Weber, 2001).

2.3 Routes of exposure

In this study occupational exposure to allergens is investigated. Workers may come in contact with allergens via the skin and mucosa, or due to inhalation.

2.3.1 Contact allergy

In contact allergy the first step is the uptake of the allergen by the epidermis. Both type I allergy (*e.g.* contact urticaria) and type IV allergy (contact eczema) may occur after exposure. Regarding contact eczema the larger the molecule the smaller the probability to be taken up by cells. Therefore, the responsible allergens are most often small molecules, not proteins. Small wounds and irritating agents creating wounds will facilitate the uptake of allergens through the skin, especially larger molecules. This is then followed by sensitisation and elicitation upon later contacts.

Multiple contact allergies (type IV allergy) may develop in susceptible persons (Carlsen *et al.*, 2008). Genetic factors play a role in the increased susceptibility, whereas cross-reactions only explain a small part of the occurrences.

2.3.2 Inhalation allergy

The proteins that are present on particles like pollen, dust, fungi and mites are the causing agent. They often have a protease activity.

Allergic rhinitis is defined as inflammation of the mucous membranes triggered by an IgE–mediated response to an extrinsic protein. The allergic sensitisation that characterises allergic rhinitis has a strong genetic component (Skoner, 2001). Patients that repeatedly come into contact intranasally with

the allergen, tend to react with an immediate response on ever decreasing amounts of allergen (priming effect).

In allergic asthma the airways become inflamed resulting in increased contractibility of the surrounding smooth muscles (bronchospasm). The lower airways become flooded with thick mucus. Asthma is caused by a combination of complex and incompletely understood environmental and genetic interactions. Epithelial injury by viruses, bacteria, tobacco smoke, air pollutants and/or oxidative stress enhances allergen passage (Galli *et al.*, 2008).
Although respiratory allergy is mostly based on type I mechanisms also type III and IV responses may occur as is the case of allergic alveolitis (*e.g*. farmer's lung and pigeon breeders lung)(see Annex).

2.4 Individual predisposition

As only a fraction of individuals exposed to allergens develop allergy, this suggests that individual susceptibility factors play an important role in the expression of clinical disease. Susceptibility to allergic disease is a complex genetic trait with many polymorphic genes involved. A person's genetic constitution, variations in genes involved in immune/inflammatory regulation, can modulate how this person interacts with these agents. But also gene mutations that affect the functioning of the epidermal barrier may be implicated.

There are important gene-environment interactions, but the underlying mechanisms of susceptibility and variability in disease expression are not completely known at present.

2.5 Treatment

Allergic disease can usually not be cured. Avoidance is the first measure to take to prevent an allergic reaction. However, in practice this is not always possible.

Pharmacological treatment is the mainstay of treatment to control symptoms (WAO, 2011). Most common medications have an antagonistic effect to block the action of allergic mediators, or to prevent activation of cells and degranulation processes. Examples are antihistamines, glucocorticosteroids and the recombinant DNA-derived humanised IgG1 monoclonal antibody, known as omalizumab (anti IgE).

For anaphylaxis the administration of intramuscular adrenaline (epinephrine) is the first-line treatment. Epinephrine is a hormone and a neurotransmitter regulating heart rate, blood vessel and air passage diameters amongst other functions.

For 100 years already allergen-specific immunotherapy (SIT) has been used as a desensitising therapy for allergic diseases (Akdis & Akdis, 2011; Larché *et al.*, 2006; WAO, 2011).
The person is gradually vaccinated with progressively larger doses of the disease causing allergen. The aim is to achieve hyposensitisation in order to reduce the symptoms occurring during the natural exposure to the allergen. This can be done with an allergenic extract or vaccine, and in recent years with recombinant allergens. The beneficial effects of immunotherapy persist for years after discontinuation. However, SIT with inhalant allergens usually provides only partial protection. Furthermore, SIT is time consuming and requires specialised care.

2.6 Conclusions

Recognizing the limitations of the summary presented here, the information in this section highlights a number of elements that need to be taken into account when considering the allergenic potential as a hazard:

- The immune-response is a complex mechanism in which a GMO or a product obtained from a GMO can influence the response in different ways.
- The risk factors for allergic disease are a person's genetics, the allergens and the environment.
- Two people may react completely different (*e.g.* show different threshold levels) when exposed under the same conditions (genetics).
- Allergy development through skin contact in most of the cases is due to small molecules. Proteins may only pass the skin when injured or compromised as in atopic individuals. In airway allergy proteins play a more important role.
- The sensitivity of the mucosa may, apart from the genetic determination, be acquired for example, during infections or exposure to irritant substances, such as diesel exhaust particles (environment).
- Actual exposure has to take place in order to develop an allergic response. The types, duration, and intensity of allergen exposure are the main factors that determine symptoms.
- The process occurs in two phases (sensitisation and elicitation). Since during sensitisation no symptoms are observed, it is hard to evaluate that an unwanted effect has occurred.
- Once sensitised, the symptoms of an allergy can be treated, but the specific part of the immune-response can usually not be switched of. In consequence, it is today for most allergens not possible to be "cured" from an unwanted sensitisation.

3 Regulatory framework

3.1 GMO legislation

At the European level CU of GMOs is regulated by Directive 2009/41/EC[1]. It lays down common measures to protect human health and the environment when working with GMOs. The Directive obliges the user to carry out a risks assessment (RA) of the CU activities. Guidance on how to perform a RA is provided in Commission Decision 2000/608/EC[2]. Among the adverse effects (hazards) that have to be considered in the RA, the potential allergenicity of GMOs is explicitly mentioned in Annex III.

Directive 2009/41/EC is transposed into national legislation in each of the Member States. In the Netherlands GMOs are regulated via the Genetically Modified Organisms Decree ('Besluit genetisch gemodificeerde organismen milieubeheer', '*Besluit*')[3] and amendments. Art. 5.1 of the Decree requires an applicant to perform a risk analysis before starting CU activities in conformity with the European Directive. The regulation implementing the Decree is the Genetically Modified Organisms Regulation ('Regeling genetisch gemodificeerde organismen', '*Regeling*')[4] and amendments that were last adapted in 2010 by the 'Wijzigingsregeling Regeling genetisch gemodificeerde organismen'[5]. References in this report refer to legislation in force at the time of the study.

Applications for GMO activities are submitted to the Ministry of Infrastructure and the Environment (*Ministerie van Infrastructuur en Milieu*, *IenM*). The Office Genetically Modified Organisms (GMO Office, *Bureau GGO*) handles the applications, checks the RA for correctness and completeness and decides on the authorisation. The Ministry is ultimately responsible for the decisions.

3.2 Workers protection

Health protection of personnel handling GMOs is also covered by a second area of legislation. The European Directive 2000/54/EC[6] specifically addresses the protection of workers from risks related to exposure to biological agents at work.
It defines biological agents as (Art.2):

> *"… micro-organisms, including those which have been genetically modified, cell cultures and human endoparasites, which may be able to provoke any infection, allergy or toxicity".*

Biological agents are classified into four risk groups: from risk group 1 without any hazard to human health to group 4 that may cause serious disease usually without effective prophylaxis or treatment available (Art. 2, elaborated in Annex III). The Directive explains the duties of the employer, namely the risks at the workplace first need to be identified and assessed; if possible the agent must be replaced

[1] Directive 2009/41/EC of the European Parliament and of the Council of 6 may 2009 on the contained use of genetically modified micro-organisms, OJ L125, 21.5.2009, p75-97.
[2] Commission Decision 2000/608/EC of 27 September 2000 concerning the guidance notes for risk assessment outlined in Annex III of Directive 90/219/EEC on the contained use of genetically modified micro-organisms, OJ L258, 12.10.2000, p43-48.
[3] Besluit genetisch gemodificeerde organismen milieubeheer van 25 januari 1990 (BWBR0004703, Stb. 1990, 53) and amendments.
[4] Regeling genetisch gemodificeerde organismen van 28 mei 1998 (BWBR0009653, Stcrt. 1998, 108)
[5] Wijzigingsregeling Regeling genetisch gemodificeerde organismen (herziening bijlage 1 en actualisering indeling handelingen in procesinstallaties) (BWBR0028026, Stcrt. 2010, 12420).
[6] Directive 2000/54/EC[6] of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work. OJ L 262, 17.10.2000, p.21-45.

by a safer alternative or the risk has to be reduced using appropriate protective clothing and protective equipment. Also, the need for information and training of workers is stressed.

In the Netherlands this is incorporated in the 'Arbeidsomstandighedenwet' ('*Arbowet*'[7]) intended to protect the health, safety and well-being of the workers in general. The implementation of the law is further supported by the 'Arbeidsomstandighedenbesluit' ('*Arbobesluit*'[8]) and the 'Arbeids-omstandighedenregeling' ('*Arboregeling*'[9]). These pieces of legislation emphasise that both employers and employees are responsible for a safe working environment and repeat that an inventory and evaluation of risks has to be made first ('*Arbowet*', Art. 5), exposure has to be prevented or reduced, and that workers have to be informed on the dangers and be instructed on the working procedures and the use of personal protective equipment both to mitigate risks ('*Arbowet*', Art. 8). The '*Arbobesluit*' (Chapter 4, Section 9) specifically addresses biological agents, cell cultures en microorganisms as in Directive 2000/54/EC.

3.3 Role of COGEM

The Netherlands Commission on Genetic Modification (Commissie Genetische Modificatie, COGEM), as the scientific advisory body to the Ministry of Infrastructure and Environment, advises on the risks for human health and the environment associated with the use of GMOs. The field of activity is biotechnology in the broad sense: from agricultural to medical applications and from research to commercial introduction, but excluding food and feed safety. Its tasks are described in the Environmental Management Act (*Wet Milieubeheer*[10], Art. 2.27).

Advice may be formulated following an application for GMOs in CU. Based on a RA COGEM may suggest containment measures additional to the standard measures. The Ministry eventually decides whether or not a permit is issued with or without extra conditions.

COGEM also informs the government on ethical and social aspects related to activities with GMOs.

COGEM assessed several application dossiers concerning GMO activities with allergens. The corresponding opinions and evaluations were consulted for the report (CGM/931020-33, CGM/981008-06, CGM/990311-06, CGM/990419-04, IG 02-063/02, IG 03-188/03 and IG 12-109/00).

[7] Wet van 18 maart 1999, houdende bepalingen ter verbetering van de arbeidsomstandigheden (BWBR0010346, Stb. 1999, 184) and amendments.
[8] Besluit van 15 januari 1997, houdende regels in het belang van de veiligheid, de gezondheid en het welzijn in verband met de arbeid (BWBR0008498, Stb. 1997, 60) and amendments
[9] Regeling van 12 maart 1997 houdende bepalingen ter uitvoering van bij en krachtens de Arbeidsomstandig-hedenwet en enige andere wetten gestelde regels (BWBR0008587, Stcrt. Suppl. 1997, 63) and amendments
[10] Wet van 13 juni 1979, houdende regelen met betrekking tot een aantal algemene onderwerpen op het gebied van de milieuhygiëne (BWBR0003245, Stb. 1979, 442) and amendments

4 Risk assessment

4.1 General principles

A RA for CU encompasses the evaluation of risks to human health and the environment and has two components, one related to the possibility of adverse effects happening, and the other related to the consequences if the adverse effect occurs. Risk is also sometimes defined as the hazard combined with the likelihood that the hazard will occur:

Risk = Hazard & Likelihood

A risk assessment typically takes a step-wise approach:

- Potential hazards are first identified based on the knowledge about the GMO (**hazard identification**).
- In the subsequent **hazard characterisation**, the potential consequences (harm), either as direct, or indirect, immediate and delayed effects, are evaluated. This involves the qualitative or, whenever possible and useful, quantitative description of the nature of the hazards and their respective accompanying uncertainties.
- Parallel to the hazard characterisation an assessment of the **likelihood** of occurrence (or exposure) of each of the identified hazards is prepared.
- Hazard and exposure characterisation lead to **risk characterisation** as the qualitative or quantitative estimate of the probability of occurrence and severity of adverse effect(s) or event(s). Often uncertainty about the severity of effects or their occurrence has to be dealt with. One way to solve this is to assume a worst-case scenario. If the risk of a worst-case scenario is found to be negligible, the risk of the 'less than worst-case' would also be negligible.
- The next step is **risk management** that aims to control identified risks and address remaining uncertainties.
- The final step is the overall **conclusion** concerning risks on the proposed activities. Whether the resulting overall risk is acceptable is the responsibility of the competent authorities, not the assessors.

Information on the recipient organism, the insert and its donor organism, and the vector that was used to modify the recipient organism will support the assessment of the potential adverse effects and their magnitude. Depending on the type of activities, their scale and duration workers will to a certain extent be exposed to the GMO. Combining these elements will lead to a risk classification of the GMO activity. In the Netherlands the '*Regeling*' comprises these rules for risk classification of GMOs in Annex 5 combining information on the type of recipient organism (Annex 1), the vector that is used (Annex 2.1) and the type of insert and its function (Annex 2.2) (Art. 7). This has been set-up to standardise and also to simplify the assessment. The classification leads to an appropriate containment level. The criteria have been devised in such a way that the containment level is adequate in the large majority of cases. The accompanying containment measures (equipment, facilities, working procedures) are listed in Annex 4. If biosafety is not adequately guaranteed using these standard classification rules, more precise and adjusted containment requirements will be formulated in the permit (Art. 7.2). In these cases COGEM is asked to assess the risks in detail and give advice on (extra) containment measures.

Activities with GM micro-organisms (GMMs) containing genetic information for harmful substances that may be active in the host, are usually classified as BSL II activities according to Annex 5, §5.2.f of the '*Regeling*'. Allergens are a special group of harmful substances, Notwithstanding, as explained in the

'*Leeswijzer bijlage 2, 5 en 8*'[11], §4.2 a gene product is defined as harmful following the properties of the protein and the genetic and physiologic context in which it is expressed (likelihood of occurrence of the hazard), The latter may be broadened to the type of activities with the allergen producing GMO.

4.2 Assessing allergenicity

Focusing on allergenicity, assessing a GMO for its potential allergenic effects is part of the first step in the risk assessment. In this respect two components are important: the nature of the recipient organism and the genetic modification (insert and vector).

4.2.1 The recipient organism

The recipient organism, *i.e.* the organism to be genetically modified, may already induce allergic effects. Species lists can be consulted that summarise information on organisms for which an allergenic potential has been noted:

- The IUIS database of the World Health Organization and International Union of Immunological Societies (WHO/IUIS[12]) is an extensive collection of organisms known to have allergenic properties including their allergens.
- Directive 2000/54/EC lists in Annex III microorganisms with their risk group including those with allergenic potential. Among the parasites are *Ascaris lumbricoides* and *Ascaris suum* both risk class 2 organisms and allergenic. Also many fungi of class 2 are known to be allergenic: *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans* var. *neoformans* (*Filobasidiella neoformans* var. *neoformans*), *Cryptococcus neoformans* var. *gattii* (*Filobasidiella bacillispora*), *Epidermophyton floccosum*, *Microsporum* spp. and *Penicillium marneffei*, and the class 3 fungus *Coccidioides inunitis*.
- Databases such as IMGT Lexique[13] provide lists of plants and animal with food allergenic properties.
- The European Academy of Allergy and Clinical Immunology lists substances, species and enzymes in relation to occupational allergies (Sastre & Quirce, 2010).
- Also, in scientific literature tables are presented of known allergenic organisms. To name only a few examples, Esch *et al.* (2001) and Weber (2001) review plants with allergenic pollen, fungi, animals and arthropods. Martel and colleagues (2010) describe microorganisms that may cause respiratory sensitisation.

If the recipient organism is mentioned on such lists, then further assessment will be required.

4.2.2 The genetic modification

The genetic modification may be an inserted gene for a new protein that may be allergenic by itself or, indirectly, by catalysing biochemical reactions that lead to the production of allergens.

4.2.2.1 Intentionally expressing an allergen

While as a general rule, one will try to avoid working with proteins that can be considered allergens, there may be particular reasons for actually seeking to express (modified) allergens. **Recombinant**

[11] http://www.ggo-vergunningverlening.nl/Vergunningverlening/Documenten
[12] www.allergen.org
[13] http://www.imgt.org/IMGTeducation/IMGTlexique/A/AllergensBiochemicalData.html

allergens, *i.e.* allergens that are produced using GMOs, are intentionally produced for research, diagnostics, and therapeutics:

- **Research** – To unravel the mechanism of an immune and allergic response recombinant allergens are produced that contain the whole or only part of the amino acid sequence. In this way for example the structural effects of the arrangement of epitopes may be studied (Chow *et al.*, 2000; Valenta & Kraft, 2001).

- **Diagnostics** - Allergenic extracts consist of complex mixtures of substances with a variation in allergenic activity and allergen composition. To improve diagnosis and allow standardisation allergens are produced using recombinant techniques (Chapman *et al.*, 2000; Seismann *et al.*, 2010). Birch pollen and latex allergens are examples of commercially available recombinant allergens for diagnostic purposes.[14]

- **Allergen immunotherapy** - Also for therapeutic purposes, recombinant allergens are produced (Focke *et al.*, 2010; Linhart & Valenta, 2005; Larché *et al.*, 2006; Niederberger *et al.*, 2004; Valenta & Niederberger, 2007). Specific immunotherapy is a desensitisation procedure reducing the sensitivity to specific allergens. The practice involves administering gradually increasing doses of the causative allergens. These recombinant wild type–based vaccines may be applied through subcutaneous injections or sublingual formulations over a period of several years. From clinical studies it appeared that more IgG is produced and the T_H1/T_H2 balance restored. The modulation of B and T cell responses, blocking antibodies, IL-10 and other cytokines also play a role in SIT using wild-type based allergens.
  To increase product safety modified molecules with reduced allergenic activity (reducing IgE-mediated side-effects) and/or increased immunogenicity are used. Fragments of the natural allergen may be applied or they may be chemically modified *e.g.* with aldehydes (so-called allergoids) or coupled to IgE suppressive adjuvants, *e.g.* aluminium hydroxide. These molecules bind MHC class II molecules, which then interact with specific T cell receptors and induce T cell tolerance, but fail to interact with IgE and therefore should not induce an allergic response. Hypoallergenic vaccines have been developed to treat *e.g.* birch, grass and Japanese cedar pollen allergies,[15]
  Also, hybrid molecules assembling the epitopes of several different allergens are being produced. Peptide carrier fusion vaccines against grass pollen allergies are currently being evaluated in phase II clinical trials.[16]

A variety of recombinant allergens are produced in heterologous hosts like *E. coli*, yeast or in eukaryotic cell lines (*e.g.* insect cells) or plants (Breiteneder *et al.*, 2001; Obermeyer *et al.,* 2004; Schmidt *et al.*, 2008; Singh & Bhalla, 2006; Wagner *et al.*, 2004; Wallner *et al.,* 2004). Intentionally working with (recombinant) allergens has the advantage that information on the allergen should be available and that accordingly the risk assessment and selection of preventive measures can be better targeted.

Types of allergens

Allergens that are frequently worked on include allergens from plant pollen, mites, cockroaches, cats, dogs and fungi (Table 1). Starting from the properties of the natural version, a judgment may be made on the characteristics of the modified allergen.

A distinction can be made between major allergens and minor allergens on the basis of the relative number of allergic patients responding to the allergen. A 'major' allergen is one to which >50% of

[14] Thermo Fisher Scientific, ImmunoDiagnostics; http://www.phadia.com/en/Allergy-Blood-Testing/ and http://www.phadia.com/en/Allergy-Blood-Testing/Laboratory-excellence/Reliable-results-with-ImmunoCap/
[15] BIOMAY; http://www.biomay.com/research-development/product-pipeline/
[16] BIOMAY; http://www.biomay.com/research-development/peptide-carrier-fusion-vaccines/

allergic patients react (based on tests for IgE-binding potency) (Chapman, 2004; Hauser *et al.*, 2010). Alternatively, 'major' may refer to the amount of a particular allergen relative to the whole spectrum of the allergen source, or to their allergenicity, *i.e.* the ability to induce a high specific IgE titre in the allergic population, leading to symptoms (Hrabina *et al.*, 2008).

Table 1 Examples of frequently used natural and recombinant allergens for research, diagnostics and therapies, and their properties

Species	Allergen	Protein function (IUIS[17])	Reference examples
Betula verrucosa	Bet v 1	pathogenesis-related protein, PR-10	Breiteneder *et al.*, 2001; Ferreira *et al.*, 1996; Focke *et al.*, 2004; Rossi *et al.*, 1996
	Bet v 2	profilin	Rossi *et al.*, 1996
	Bet v 4	calcium-binding	Twardosz *et al.*, 1997
Dactylis glomerata	Dac g 1	β-expansin	Van Oort *et al.*, 2004
Lolium perenne	Lol p 1	β-expansin	Perez *et al.*, 1990
	Lol p 2	belongs to expansin B subfamily	Van Ree *et al.*, 1994a
	Lol p 3	belongs to expansin B subfamily	
	Lol p 5	Ribonuclease activity	Swoboda *et al.*, 2002
Malus domestica	Mal d 1	pathogenesis-related protein, PR-10	Vanek-Krebitz *et al.*, 1995
Felis domesticus	Fel d 1	uteroglobin	Saame *et al.*, 2005; Van Ree *et al.*, 1999; Versteeg *et al.*, 2011
Canis familiaris	Can f 1	lipocalin	Konieczny *et al.*, 1997
	Can f 2	lipocalin	
Dermatophagoides pteronyssinus	Der p 1	cysteine protease	Jacquet, 2013; Kauffman *et al.*, 2006; Walgraffe *et al.*, 2009
	Der p 2	MD2-like lipid binding protein	
	Der p 5	lipid binding protein	
	Der p 7	lipid binding protein	
Blattella germanica	Bla g 2	Inactive aspartic protease	Wünschmann *et al.*, 2005
Vespula vulgaris	Ves v 1	phospholipase A1	Seismann *et al.*, 2010
	Ves v 5	cysteine-rich venom protein	
Aspergillus fumigatus	Asp f 13	alkaline serine proteinase	Chow *et al.*, 2000

A second element to be taken into account is the potential for cross-reactivity. Proteins involved in general vital functions, therefore widely found in plants and animals, may have similar structures because of the related function leading to cross-reactivity. Major as well as minor allergens may be present in unrelated species and are called panallergens (Hauser *et al.*, 2010). Panallergens comprise only a few protein families, including above all profilins, and also polcalcins, Bet v 1 homologues and non-specific lipid transfer proteins. Examples are listed in Hauser *et al.* (2010).

Examples of wild-type allergens

Grass major pollen allergens Lol p 1 and Lol p 5 belonging to group 1 and group 5 grass pollen allergens are 'recognised' by 90-95% and 65-85% of grass-pollen-allergic patients and are classified as having a high prevalence and potency (Hrabina *et al.*, 2008). They have homologues in other species with similar properties, hence the grouping. Group 1 allergens show a high sequence homology between Pooideae species. Both groups display strong cross-allergenicity between Pooideae.

[17] http://www.allergen.org/search.php

Profilins, highly cross-reactive panallergens, are structurally conserved eukaryotic proteins found in trees, grasses, weed pollens, fruits, vegetables, nuts and latex (Hauser *et al.*, 2010; Kazemi-Shirazi *et al.*, 2002). They are present as cytoskeletal proteins. Therefore, profilin-sensitised patients may show allergic reaction to several unrelated allergen sources or are at risk of developing allergic symptoms.

The major allergen of birch pollen is represented by Bet v 1, with more than 95% of birch pollen-allergic patients showing IgE reactivity to Bet v 1 (Valenta *et al.*, 1991). Related proteins occur in pollen of other trees of the *Fagales* order, but also in somatic plant tissues like fruits, vegetables and spices, *e.g.* apple Mal d 1. Therefore, patients may be sensitive to food allergens as well (cross-reactivity).
Bet v 4 is a calcium-binding protein (polcalcin) containing two binding sites for calcium, termed EF-hands. Again, homologues are present in pollen from other plant species that may cause cross-reactivity (Kazemi-Shirazi *et al.*, 2002; Valenta *et al.*, 2010). Expression is restricted to pollen.

The major cat allergen Fel d 1, a glycoprotein, consists of 2 heterodimers each with two chains encoded by different genes (Versteeg *et al.*, 2011). Depending on the technique 80-90% of the total IgE response against cat allergens is shown to be directed to this allergen (van Ree *et al.*, 1999).

Can f 1 and Can f 2 are major and minor dog allergens, respectively. Both are secreted by the tongue epithelial tissue and belong to the lipocalin family of small ligand-binding proteins (Konieczny *et al.*, 1997). On average they account for 70% and 23% respectively of IgE binding to dog dander extracts.

In an industrial setting the most prevalent enzymes that may cause occupational allergy are derived from the genus *Aspergillus*: they include α-amylases, xylanases, and cellulases (Green & Beezhold, 2011; Martel *et al.*, 2010).

Some proteases not only have an allergenic potency, they also directly affect epithelial integrity and permeability, mast cell degranulation and cytokine release from the respiratory epithelium cells. (Kauffman *et al.*, 2006; Matsumura, 2012; Stewart *et al.*, 1993). Examples are Der p 1, a cysteine protease from the house dust mite *Dermatophagoides pteronyssinus*, proteases in pollen of many plant species, Asp f 13, an alkaline serine proteinase from *Aspergillus fumigatus*, etc. (Baur, 2005; Jacquet, 2013; Kauffman *et al.*, 2006).

Examples of allergens in research
The above listed types of allergens attract most attention of researchers and medical professionals, because of their importance for human health. They may be produced as recombinant wild-type based allergens. At the same time they have a high potential of causing adverse effects on personnel. However, modifications may alter their properties. Modified allergens are used in diagnostics (so-called marker allergens) and as vaccines in allergen immunotherapy. Fig. 1 summarises the various possibilities for modifications. They are further explained below.

By introducing point mutations, deleting parts of the sequence, by fragmenting, oligomerising, chemically modifying and by fusing allergen variants, so-called ‘recombinant hypoallergenic allergen derivatives’ are made. Such hypoallergenic derivatives may reduce IgE reactivity and allergenic activity, but they retain immunogenicity and T cell reactivity of the wild-type allergens (Focke *et al.*, 2010; Linhart & Valenta, 2005; 2012; Valenta *et al.*, 2010). The conformational IgE epitopes are disturbed or the amino acids required for IgE binding are mutated. This means that protecting, allergen-specific IgG antibodies may still be induced. They recognise the wild-type allergen but block the recognition of the wild-type allergen by IgE.

Another modification is the absence of glycosylation. For diagnostic purposes non-glycosylated recombinant allergens are preferred to avoid non-relevant cross-reaction with carbohydrate epitopes (Valenta *et al.*, 2010).

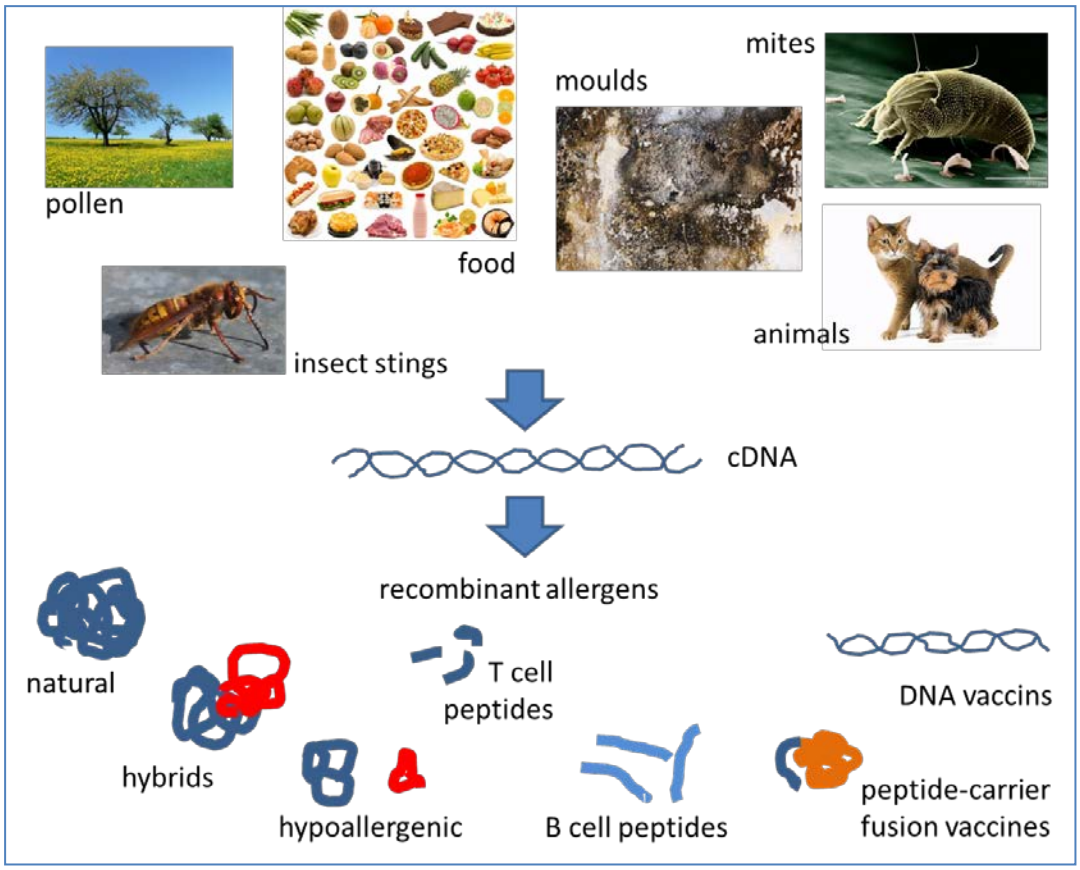

Fig. 1 Types of allergy vaccines (Modified from Linhart & Valenta, 2005)
Using allergen-encoding cDNAs from various sources, recombinant allergens for diagnosis and therapy can be obtained, and engineered. Allergen-encoding RNA is first isolated and converted into cDNA. These cDNAs are inserted into expression systems and recombinant wild-type allergens are produced. The DNA sequences may be modified into a variety of allergy vaccines including hybrid molecules, hypoallergenic derivatives, T cell and B cell peptides as well as DNA vaccines and peptide-carrier fusion vaccines.

Site-directed mutagenesis may induce point mutations altering the IgE binding epitope thereby reducing or completely avoiding recognition (Spangfort *et al.*, 2003). The same effect can be obtained via DNA shuffling followed by selection for low binding to IgE (Gafvelin *et al.*, 2007).

Allergen-derived T cell epitopes were developed with the idea that they are too small to be recognised by allergen-specific IgE, thereby eliminating IgE-mediated side-effects (Focke *et al.*, 2010). They bind to the T cell receptors of allergen-specific T cells and should induce T cell tolerance. However, clinical studies revealed that late adverse events may occur that are non-IgE-mediated and dependent on the dose. They result from the MHC-dependent activation of allergen-specific T cells (Linhart & Valenta, 2005; Valenta *et al.*, 2010). However, these small sized T cell–reactive peptides do not induce relevant allergen-specific IgG responses (Valenta *et al.*, 2010).

Allergen-derived B cell peptides are larger than T cell epitope peptides. They contain some amino acids exposed to the surface of the natural allergen, but are selected for lack of allergenic activity which is due to the loss of conformational integrity (Linhart & Valenta, 2005; Valenta *et al.*, 2010). They too are able to induce IgG antibodies which recognise the wild-type allergen inhibiting allergen-induced basophil degranulation and to reduce the boost of IgE memory responses.

Likewise, folding variants due to mutations or the assembly of oligomers of hypoallergenic fragments can be produced for immunotherapy because of their reduced allergenicity while retaining

immunogenicity. Also, hybrid molecules are developed stably combining epitopes of various allergen sources to facilitate the manufacturing of allergy vaccines. They may exhibit unexpectedly useful immunological properties, such as, again, increased immunogenicity and reduced allergenic activity (Focke *et al.*, 2010; Linhart & Valenta, 2005).

Another type of modification is represented by the CpG-modified allergens (Focke *et al.*, 2010; Valenta *et al.*, 2010). Unmethylated CpG motifs act as immune adjuvants. CpG motifs appear in microbial genomes, but are rare in vertebrate genomes. These CpG pathogen-associated molecular patterns are recognised by the pattern recognition receptor Toll-Like Receptor 9 that is expressed only in B cells and dendritic cells in the blood. CpG motifs promote the induction of T_H1 and pro-inflammatory cytokines and support the maturation/activation of professional APCs. The effect of these immuno-stimulatory DNA sequences can be dramatically enhanced by direct linkage to the allergen.

The most recent type of allergen modification for immunotherapy are the peptide-carrier fusion vaccines (Focke *et al.*, 2010; Valenta *et al.*, 2010). Peptides are derived from the surface of various allergens. These amino acid stretches are located closely to an IgE epitope or overlap with it, but do not contain T cell epitopes. They themselves show no or only minimal IgE reactivity. When coupled to a non-allergenic but immunogenic carrier molecule, a carrier-specific T cell response is induced against the carrier and anti-peptide IgG antibodies are produced. When exposed to this type of molecules, no allergen-specific T cells are activated. Viral capsid proteins are used for the purpose, as they display an immuno-modulatory activity towards a T_H1 response.

In Table 2 an overview of characteristics in relation to safety for use in immunotherapy of the various types of modifications is given. The ideal molecule would induce the production of protective antibodies (IgG) and would have no (-) or reduced IgE and T cell reactivity or side-effects.

Table 2 Strategies for protein- and peptide-based immunotherapy

Approach	Induction of protective antibodies	IgE reactivity	Possible IgE-mediated side-effects	T cell reactivity	Possible T cell-mediated side-effects
Recombinant wild-type allergens	+	+	+	+	+
Point mutations / DNA shuffling to reduce IgE activity/ fragmentation / deletion	+	reduced*	reduced	+	+
Allergen-derived T cell epitopes	-	-	-	+	+
Allergen-derived B cell epitopes	+	reduced	reduced	-	-
Folding variants / oligomers	+	reduced	reduced	+	+
Epitope hybrids	+	reduced	reduced	+	+
CpG conjugated allergens	+	reduced	reduced	+	+
Peptide-carrier fusion vaccines	+	-	-	-	-

Table derived from Focke *et al.*, 2010; Linhart & Valenta, 2005; Valenta *et al.*, 2010.
*reduced reactivity compared to wild-type

Examples of allergens in industry

Other protein allergens that are known respiratory sensitizers are several industrial enzymes, often handled in large quantities and therefore receive special attention by the legislator. They are listed in Annex I of Council Directive 67/548/EEC[18] relating to the classification, packaging and labelling of dangerous substances and have to be labelled with risk phrase R42: 'May cause sensitisation by inhalation'. This list has been regularly updated through Adaptations to Technical Progress.

R42 is replaced by H334 in Regulation (EC) No 1272/2008[19] that will come into force by June 1, 2015.
The respiratory sensitisation, category 1 enzymes (Annex I, section 3.4: Respiratory or skin sensitisation) that have to be labelled with hazard statement H334 ('May cause allergy or asthma symptoms or breathing difficulties if inhaled') are: β-glucosidase, cellulase, exo-cellobiohydrolase, bromelain (proteases), papain (papaya proteinase I), ficin (cysteine endopeptidase), pepsin A (endopeptidase), rennin (chymosin), trypsin, chymotrypsin, subtilisin (serine protease), microbial neutral proteinase, protease, α-amylase and other amylases.
There is no mentioning of enzymes that may cause an allergic skin reaction (H317: 'May cause an allergic skin reaction').

4.2.2.2 Influencing the expression of an endogenous allergen

The host organism already may contain known endogenous allergenic proteins. Due to the genetic modification the expression level may be altered, *i.e.* increased or decreased. However, as the allergen is likely to remain the same, this will rarely influence the risk analysis. The main reason is that there is no linear relationship between the amount of an allergic protein that an allergic person is exposed to and the severity of the disease. Also, threshold levels may be very low, hence solely presence already may result in an adverse reaction. As with food allergens total avoidance is the best protection (Goodman *et al.*, 2008).
If necessary, proteomics methods may be used to assess the potential increase of the intrinsic allergenicity of the GMO as an unintended effect of the genetic modification.

4.2.2.3 Expressing other proteins (non-intentional allergens)

To assess the potential allergenicity of the newly-expressed protein(s) the 'Weight-of-evidence approach' may be used (Fig. 2). This stepwise, case-by-case approach, standardly applied when assessing GM food, should rely upon various criteria used in combination, since no single criterion is sufficiently predictive on either allergenicity or non-allergenicity as explained in Codex Alimentarius Guideline CAC/GL 45-2003, Annex 1, and Codex Alimentarius Commission, Alinorm 03/34 (2003). In such cases an assessment based on a decision tree as proposed by FAO (2001) was found to be too rigid (Goodman *et al.*, 2008; Ladics, 2008).
Although developed for assessing allergenicity in food derived from recombinant-DNA plants, the principles may serve allergenicity assessment of proteins in general (Fig. 2). The different steps include examination of:

- the gene source,
- the sequence to check for homology with known allergens,
- the physicochemical properties, supplemented with
- immunologic analyses, if necessary.

[18] Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. OJ L196, 16.8.1967, p. 1–98 (English edition p. 234–256).
[19] Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. OJ L353, 31.12.2008, p.1-1355.

It should be stressed however that having a favourable outcome, is a strong indication, but no absolute guarantee for absence of potential for allergenicity.

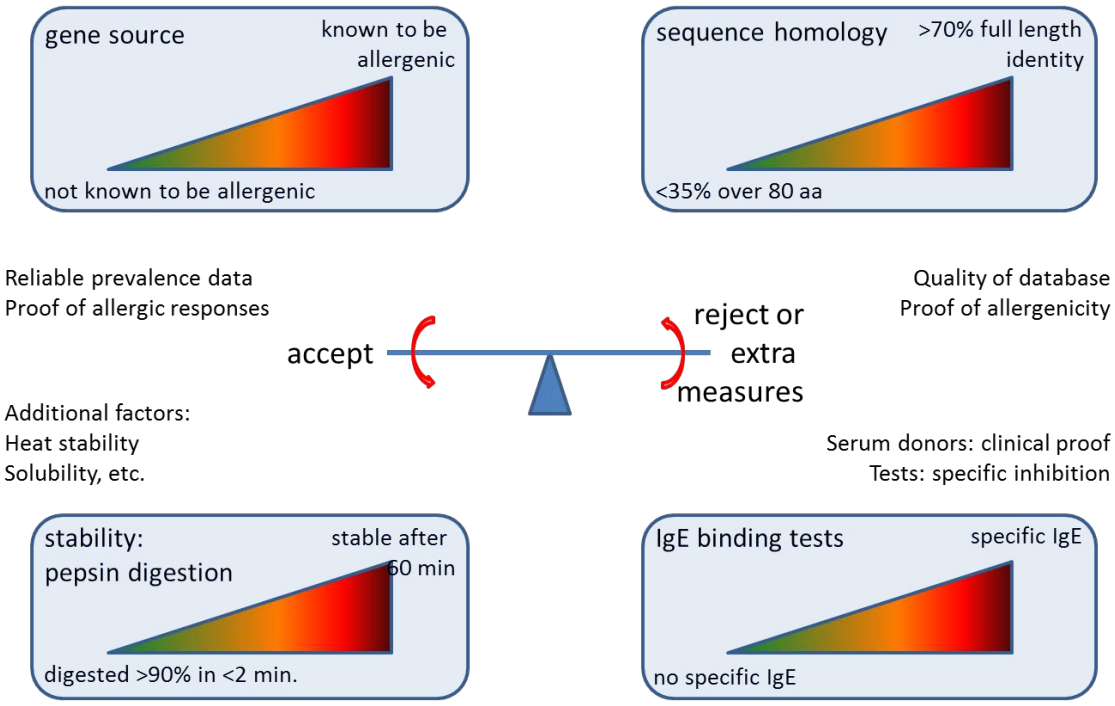

Fig. 2 Schematic interpretation of the weight-of-evidence approach described by the Codex Alimentarius Commission (Modified from Goodman *et al.*, 2008)

Gene source

Regarding the gene source the characteristics of the donor organism are important. Any indication of an IgE mediated oral, respiratory or contact allergy should trigger further investigation to demonstrate or exclude the gene's role therein. Again, the lists mentioned in 4.2.1 may be of help, followed by further literature searches. Weber (2001) additionally gives an overview of the known antigens in these organisms. Related species may share antigens: the closer their phylogenetic relationship the greater similarities and the more shared antigens (Weber, 2001). This knowledge may be taken into account when assessing potential allergenicity and cross-reactivity.

***In silico* tests**

In silico bioinformatic approaches will give insight in the amino acid sequence, protein structure and post-translational processing of the inserted gene that potentially result in allergenicity. Guidance has been developed by Codex Alimentarius (2003), the FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (2001) and the Working Group on "The Assessment of allergenicity of genetically modified foods" of the EFSA GMO Panel (2010). Again, although focusing on food allergens these guidelines may be of value.

The FAO/WHO Expert Consultation group proposed a method with 2 elements for allergenicity/IgE-cross-reactivity assessment: a sequence match with known allergens of 6 consecutive amino acids and an identity of more than 35% over an 80-amino acid window to trigger further analysis (FAO/WHO, 2001). The 6 amino acid match may identify potential linear IgE epitopes or possibly also T cell epitopes, whereas the second criterion aims at detecting potential conformational IgE-epitopes. Indeed, also tertiary structure is important. In the absence of similarity in folding with allergens, protein cross-reactivity is virtually excluded (Aalberse, 2000). While this protocol only gives an indication -

false-positives as well as false-negatives are inherent to these approaches– they may be supplemented with other tools as they become available (EFSA, 2010).
However, it should be noted that these *in silico* bioinformatics approaches are not appropriate for the assessment of the *de novo* sensitisation potential of a newly expressed protein. They only provide an indication on the possible IgE-cross-reactivity with known allergens (EFSA, 2010).

Allergen databases used for the initial screening are presented in Table 3. The alignment method has its limitations as it depends on the presence of known allergens in databases. A negative result (no matches found) does not necessarily mean no allergenicity. Furthermore, it is difficult to detect non-contiguous epitopes that bind specifically with IgE antibodies.

Also, epitope databases that relate to allergy are available, *e.g.* the Immune Epitope Database (IEDB) (Vaughan *et al.*, 2010). Apart from food allergen epitopes, the largest group, also epitopes defined for aeroallergens and contact allergy are represented.

Table 3 Allergen, protein and epitope databases

Database	URL
IUIS Allergen Nomenclature Sub-Committee	http://www.allergen.org/Allergen.aspx
AllergenOnline (FARRP)	http://www.allergenonline.org
IMGT allergen page	http://www.imgt.org/IMGTeducation/IMGTlexique/A/AllergensBiochemicalData.html
The Allergen Database (CSL)	http://allergen.csl.gov.uk
Allergen Database for Food Safety (ADFS)	http://allergen.nihs.go.jp/ADFS/
Allergome	http://www.allergome.org
Structural Database of Allergenic Proteins (SDAP)	http://fermi.utmb.edu/SDAP/sdap_src.html
AllerMatch	http://www.allermatch.org/
Allfam database	http://www.meduniwien.ac.at/allergens/allfam/
Swiss-prot protein knowledge base	http://www.uniprot.org/docs/allergen.txt
EMBL-EBI, European Bioinformatics Institute	http://www.ebi.ac.uk/
Immune Epitope Database (IEDB)	http://www.immuneepitope.org/
Epitome	http://www.rostlab.org/services/epitome/
TEPITOPEpan / MHC peptide binding prediction	http://www.biokdd.fudan.edu.cn/Service/TEPITOPEpan/TEPITOPEpan.html

The specific folding of proteins (tertiary or three-dimensional structure) often determines the allergenic activity. Whereas T cell epitopes are small peptides resulting from the proteolytic processing of allergens, IgE epitopes are surface-exposed allergen structures. IgE epitopes may be assembled by short stretches of amino acids from distinct locations on the protein that are brought together through folding of the allergen molecule (Valenta & Kraft, 2001). However, the different allergens do not have a common structural frame associated with their ability to elicit an allergic immune response. In general the structural similarity, causing cross-reactivity, reflects the phylogenetic relationship between

species. Nevertheless, groups of protein allergens of unrelated organisms may be identified with both conserved three-dimensional structures and homologous sequences. Examples are the highly conserved profilins and the Bet v 1 family. As a result they exhibit a high degree of IgE cross-reactivity due to their structural resemblance (EFSA, 2010).

Next to peptides also carbohydrates may act as epitopes, although post-translationally derived, and may be shared by taxonomically unrelated species (EFSA, 2010). Therefore, IgE responses directed towards plant N-glycans show high cross-reactivity. In this respect the host organism of the genetic modification is important. The post-translational modifications by eukaryotic vs. prokaryotic systems may have an impact on the allergenic potential of the protein.

Physicochemical properties

As for food allergens, methods to assess the stability of the three-dimensional structure, may provide an indication of potential for allergenicity.

Food proteins that are susceptible to enzymatic degradation, acids, heat and processing, may be degraded very quickly and are unlikely to sensitise and/or elicit susceptible persons. Likewise, size, stability and solubility of the intact protein would be relevant factors for airborne allergens.
Size and solubility affect the potential for mucosal transport. Structural stability (resistance to proteases) influences the binding efficacy between the allergen and IgE. Structural stability also preserves the functional properties: proteases and nucleases may damage tissues.

Posttranslational modifications may affect allergenicity by (Aalberse, 2000; FAO, 2001):

- inducing new epitopes;
- affecting solubility, stability, size, and susceptibility toward proteolysis;
- affecting uptake and processing by antigen-presenting cells.

Especially effects of glycosylation are important in this respect.

Methods to investigate the structure like X-ray crystallography and NMR analysis are rather costly and time-consuming and need sufficient amounts of proteins. Circular dichroism spectroscopy, often used to check recombinant allergens, may provide an alternative (Verdino & Keller, 2004).

Immunologic analyses

- ***In vitro* IgE binding tests**

If bioinformatics studies show that a transgenic protein has relevant similarities to a known allergen, further testing is needed. Specific serum screening is essentially an evaluation of the reactivity the protein with IgE antibodies in the sera of patients with known allergies. Some IgE-binding assays that may be used to assess cross-reactivity are: Radio or Enzyme Allergosorbent Assay (RAST or EAST), Enzyme Linked Immunosorbent Assay (ELISA) and electrophoresis followed by immunoblotting with specific IgE-containing sera (EFSA, 2010; Poulsen, 2001). The specificity and affinity of the IgE response may however vary between individuals. Also, it may be difficult to find relevant sera in sufficient quantities.

When a newly expressed protein is not found to be homologous to allergens in databases, it may still cross-react with allergens not yet in the databases. To minimise the risk of introducing a transgenic protein that is homologous to an “unknown” allergen targeted serum screening is used (EFSA, 2010; FAO, 2001). For that purpose sera are used with IgE directed towards allergens that are broadly related to the source of the transgenic protein. For example if the recombinant protein is derived from a monocot, serum samples from patients with high levels of IgE antibodies to monocot allergens such as grass and rice may be used.

- **Cell-based tests**

Human and animal cell-based systems exist to test the binding capacity and the functionality to act as an allergen. The basophil degranulation assay is highly sensitive and specific. It assesses the capacity to provoke degranulation. Sensitised basophils are challenged with a protein that, when cross-linking surface-bound specific IgE, will cause histamine release from the cell. This may even be quantified and dose–response curves obtained (Poulsen, 2001). This test can be standardised using cell lines transfected with the human FcεRI receptor, such as rat basophil leukemia cells (EFSA, 2010; FAO, 2001; Poulsen, 2001). However, this test cannot predict the sensitising capacity of a (recombinant) molecule.

Also, the synthesis of leukotrienes in basophils may be monitored (Poulsen, 2001). Models used for respiratory sensitisation include primary airway epithelial cells, alveolar cells lines, and bronchial cell lines, alveolar macrophages, dendritic cells etc. (Martel *et al.*, 2010). Problems concern *e.g.* availability and donor variation. Moreover, since an allergic reaction involves a complex interaction of many cell types, a single cell type may at best only partly demonstrate an effect.

More cell systems (*e.g.* T cell, co-culture of several cell types) are being developed (EFSA, 2010, Martel *et al.*, 2010).

- ***In vivo*, animal models**

Animal models may assess two processes: either they are to study the potential of a novel protein for *de novo* sensitisation, or for elicitation of an allergic reaction in animals previously sensitised to a cross-reacting protein. Examples are the Magnusson Kligman Guinea Pig Maximisation Test and the Buehler Guinea Pig Test to assess for skin sensitisation by chemicals (cell-mediated) (OECD guideline 406), now replaced by the murine local lymph node assay (Anderson *et al.*, 2011; OECD guideline 429). However, this method is not suitable for protein testing (IgE-mediated) (Gezondheidsraad, 2008).

In general, shortcomings of animal models are that they are not able to completely mimic a potential human response. Therefore, no single model can provide definite conclusion on the allergenicity of a novel protein (EFSA, 2010). Codex Alimentarius (2003) stresses the need to use scientifically validated test methods and mentions animal models as potential future test methods. For food safety testing EFSA (2010) concludes that 'none of the developed animal models is currently sufficiently evaluated, validated and widely accepted'. To date there is no thoroughly validated method to induce and detect respiratory allergies in an animal model (Deutsche Forschungsgemeinschaft, 2012).

- ***In vivo*, human**

The classical skin prick tests may be used to test for sensitivity to inhaled allergens (IgE-mediated). Scratching or puncturing the skin introduces the protein to mast cells that are sensitised with specific IgE. They are subsequently activated via allergen cross-linking of this IgE and release histamine causing a wheal and flare reaction of the skin. Blood testing is another way to test, but, again, only detects IgE allergens and does not work for every possible allergen. To test the potential of a protein for type IV hypersensitivity (*e.g.* contact allergy), patch tests are only useful once sensitisation has occurred. Again, these tests cannot predict the risk of sensitisation.

Adjuvanticity

To test for adjuvant activity of the newly expressed protein, tests exist that may detect part of the mechanisms using *in vitro* cell systems (EFSA, 2010). When a protein is known to functionally or structurally resemble a known strong adjuvant or to belong to a class of proteins known often to have allergy adjuvant activity, this should be further investigated. However, there is no definite test -*in vitro* or *in vivo*- for adjuvanticity.

4.2.3 Conclusions

- In order to evaluate the allergenic potential of a GMO or its expressed proteins, both the recipient organism and the genetic modification must be considered.
- When the genetic modification intends to express an allergen, the analysis can be targeted to the specific product.
- Allergens may be grouped in major allergens and minor allergens on the basis of the relative number of allergic patients responding to the allergen.
- Allergens may be modified resulting in modified properties.
- Influencing the expression level of an endogenous allergen will not alter the RA, due to low thresholds and non-linear dose-response relationships.
- Other expressed proteins may be analysed for their potential allergenic nature by means of the weigh-of-evidence approach using:
    - background information on the gene source,
    - bioinformatics,
    - tests for physicochemical properties, and
    - immunologic analyses.

  However, allergenic potential cannot be fully predicted.
- Developing *in vitro* and *in vivo* tests meets difficulties on representativeness and reliability.

Dealing with CU experiments often means that the research is still in its early phases. Performing all proposed tests in this section would bring an extra cost that is mostly not in proportion to the costs of the planned activity and to the potential risk associated with it. However, the computational screening for potential allergenicity or IgE-cross-reactivity is both convenient and inexpensive, and is therefore recommended to be conducted. When a GMO or derived product is intended for marketing, more elaborate tests may be necessary, as prescribed by the product-related legislation.

4.3 Exposure analysis

Allergen exposures in laboratory, greenhouse and animal facility conditions are mostly limited to contact and inhalation. Ingestion can occur when accidentally material is swallowed *e.g.* after touching the mouth. For exposure to occur different conditions need to be fulfilled:

- The introduced gene must be expressed. Using eukaryotic promoters in a prokaryotic cell will prevent (or at least significantly reduce) expression (vector construction, transformation, transfection to host cells, etc.).

- The production of a ‘functional’ allergen often includes correct folding and posttranslational modifications (*e.g.* disulfide-bond formation or glycosylation). This means that the corresponding genes need an eukaryotic expression system. However, also incomplete proteins and peptides may have sensitisation and/or elicitation potency.

- The protein needs to be exposed or excreted. Examples are integration in the cell membrane or cell wall, or excretion into culture medium (transit peptides, excretion signals). Proteins that stay inside the cell will only pose a risk when the cell is ruptured. This occurs mainly during protein harvest and purification, but background levels may be attributed to release during normal cell degradation.

When considering CU of GMOs, any release in the environment of viable organisms is expected to be prevented. Therefore exposure of the wider environment to (potential) allergens expressed by these GMOs is not considered in this report.

4.3.1 Thresholds

4.3.1.1 Attempts to establish thresholds

Allergens are otherwise harmless substances, that are recognised by some individuals as harmful. In the immune system a complex regulating mechanism with feedback loops protects the human being from harmful effects from pathogens and foreign substances. Only when this mechanism is not able to cope with the amount of allergens, it may be disturbed leading to an allergic reaction. This amount is the threshold value for that particular allergen, below which no adverse effect is expected.

The first obstacle in determining a threshold in relation to allergens is the definition of an adverse effect. Sensitisation by itself does not show disease symptoms. However, it is a critical episode and a precondition to develop allergic disease. Therefore, the Dutch Health Council ('Gezondheidsraad') advises to use the occurrence of sensitisation to develop recommended exposure limits and thresholds (Gezondheidsraad, 2008).

Another difficulty in determining this value, unlike for toxins, is its variability among persons. While most people will not show any reaction to a given allergen, some will, in varying degrees depending on various elements, among which the concentration or amount of the substance and the duration and manner of exposure. Variation is even noticed within an individual over time (Taylor *et al.*, 2009).

Another difference with toxins is that the relation between dose and effect may not be linear (Custovic & Woodcock, 2001), although it has recently been discussed (FARRP) that also for allergens to some extent "the dose makes the poison" ("*Dosis sola facit venenum"*, Paracelsus, 16th century). Still, the dose-response relationship between allergen and disease may be different for different allergens. Examples are the almost linear curve for house dust mite, and the bell-shaped relationship for cat (Custovic & Woodcock, 2001). The latter suggests that high exposure to cat allergen has a protective effect by modifying the T_H2 response to suppress IgE production while maintaining or increasing IgG and IgG4 production (Erwin *et al.*, 2005; Woodfolk, 2005).

With two distinct processes involved in the development of allergy, sensitisation and elicitation, both will presumably have different dose-response relationships (Taylor *et al.*, 2009).

In analogy with food allergens, a threshold can be defined as "the maximum amount of an allergenic protein that can be tolerated without producing any adverse reaction" (FARRP). An individual threshold is the maximum amount of an allergen that can be tolerated by a specific allergic individual. A population threshold is the maximum amount of an allergen that can be tolerated by the entire population (or a representative sub-population) of individuals with a specific type of allergy. The challenge is to find a representative sub-set of sensitive persons. Even then the resulting dose is not a safe dose as there are always some individuals who develop sensitisation or symptoms well below these threshold values (FARRP; WAO, 2011). Therefore, to be on the safe side, sensitised symptomatic individuals are often advised to avoid the allergen totally. Nevertheless, the population threshold has a value for comparing allergenicity of substances.

Individual and population thresholds can be expressed as NOAEL (no observed adverse effect level) or LOAEL (lowest observed adverse effect level). These threshold doses for elicitation may be determined through double-blind placebo-controlled (food) challenge studies (Crevel *et al.*, 2008; Taylor *et al.*, 2009). Standardisation is necessary to be able to compare results. However, as some patients already react to the lowest dose, no NOAEL can be established. In terms of representativeness the selection of patients in relation to the overall allergic population is another difficulty (sensitivity, age, medical background, etc.).

The Key Events Dose-Response Framework is proposed by Taylor *et al.* (2009) to establish elicitation thresholds for food allergens. This analytical approach is based on clinical observations for each of the key steps in the biological process in order to facilitate examination of and to understand the various factors that determine variability.

The food labelling regulations in the USA are based on the existence of threshold levels below which it is unlikely that a food allergic individual would experience an adverse effect (FDA, 2006). However, for these threshold levels to establish, again an understanding of the dose-response relationship between the ingestion and the elicitation of an adverse response is needed. Nevertheless, dose escalation studies are performed, again pointing to the variability between individuals. It was shown that differences of a factor of 10^6 exist in eliciting doses from the least sensitive to the most sensitive individuals (FDA, 2006 and references therein). Several approaches are discussed, among which the Risk Assessment-Based Approach, determined as "the most scientifically rigorous, providing insight into the level of risk associated with specific exposures and the degree of uncertainty inherent in the risk estimate" and "is preferred when a biological threshold cannot be justified scientifically". However, it is acknowledged that data may be lacking.

For food allergens, attempts for listing thresholds are found in:

- European Voluntary Incidental Trace Allergen Labelling (EU-VITAL)[20] defining harmonised action levels based on clinical thresholds for the labelling of allergens.
- FAO, 2001, The Prevalence of Allergens in Foods and Threshold Levels for Sensitization.
- FDA, 2006, Approaches to Establish Thresholds for Major Food Allergens and for Gluten in Food
- ILSI[21]

For contact and airway allergens lists for exposure limits or classifications are available mainly for chemicals:

- Health and Safety Executive. EH40/2005 Workplace exposure limits. Containing the list of workplace exposure limits for use with the Control of Substances Hazardous to Health Regulations 2002 (as amended). Health and Safety Executive, the United Kingdom; 2011.
- WHO. Criteria for classification of skin- and airway-sensitizing substances in the work and general environments. Copenhagen: 1997. Four classes for sensitising substances are proposed with classification criteria depending on the available evidence:
    - class I: inducer of specific airway or contact hypersensitivity;
    - class II: probably an inducer of specific airway or contact hypersensitivity;
    - class III: not classifiable; and
    - class IV: not an inducer of specific airway or contact hypersensitivity.
- German Research Foundation ('Deutsche Forschungsgemeinschaft') List of MAK and BAT Values 2012: Maximum Concentrations and Biological Tolerance Values at the Workplace[22]. For airway and contact allergens no values are mentioned. Three classes are presented:
    - substances with sufficient evidence for allergenic effects;
    - substances that are probably allergenic;
    - substances for which an allergenic effect cannot be excluded.
- The Dutch Expert Committee on Occupational Safety of the Health Council ('Gezondheids-raad') advises on legally binding Occupational Exposure Limits (OELs) (Grenswaarden Stoffen op de Werkplek[23]) for hazardous substances. For protein allergens no values are set.
- The Japan Society for Occupational Health (Yano, 2012).

[20] http://www.eu-vital.org/en/home.html ; http://www.eu-vital.org/en/home.html
[21] http://www.ilsi.org/europe/pages/tf_foodallergy.aspx
[22] http://onlinelibrary.wiley.com/book/10.1002/9783527666034
[23] http://www.ser.nl/nl/taken/adviserende/grenswaarden.aspx;
http://wetten.overheid.nl/BWBR0008587/BijlageXIII/geldigheidsdatum_27-06-2013

Some data are available on exposure-response relationships of sensitisation to enzymes in workers in industrial settings (Baur, 2005). A positive association between the air concentration of enzymes, the duration of exposure and the frequency of sensitisation can be found. Studies reveal that that there is a wide range in sensitisation potency between various allergens (Heederik *et al.*, 1999). Sensitisation to rat urinary allergens (laboratory animal workers) happens in the pg/m^3 range, to fungal α-amylase (bakery workers) in the ng/m^3 range, whereas sensitisation to wheat, pig and cow proteins (farmers) occurs in the µg/m^3 range.

Today, *Bacillus subtilis'* subtilisin, a serine endopeptidase, is the only enzyme for which the American Conference of Governmental Industrial Hygienists (ACGIH) has established a threshold limit value (60 ng/m^3) (Green & Beezhold, 2011; Martel *et al.*, 2010). This ceiling level should not be exceeded during any time of the working exposure.

The Dutch Health Council concluded that in practice for most allergens no threshold level can be established below which no sensitisation will occur (Gezondheidsraad, 2008). The exposure range that can be measured for airborne allergens in studies may not include a threshold or may have a threshold near the lower range limit. The former necessitates extrapolation to the lower end, the latter experiences difficulties in the interpretation of shown effects. In both cases, without a clear cut-off, uncertainty is introduced in a way that a reliable threshold level cannot be determined.

Likewise, the German Research Foundation (Deutsche Forschungsgemeinschaft, 2012), making a comment in general, states that it is still not possible to determine generally applicable threshold levels either for the sensitisation or for triggering the allergic reaction in an already sensitised person. The concentrations required for elicitation are generally lower than those required for sensitisation. The likelihood of induction increases with the concentration of the allergen to which an individual exposed.

4.3.1.2 Proposal for classification

In the absence of a straightforward system to establish thresholds for allergens, one may only discriminate between allergens, non-allergens and uncertain cases. Following the classification of the WHO for chemical substances (WHO, 1997) the authors of this report suggest using four groups depending on the amount of evidence for allergenicity (as indicated by sensitisation):

1) Known allergens (sufficient evidence);
2) Probably allergenic (some indications present);
3) Proteins for which allergenicity cannot be excluded (ill-defined studies);
4) No indications of allergenicity.

In analogy with WHO:

- 'sufficient evidence' for protein sensitizers may be described as data obtained in clinical studies in a representative population of patients that are performed according to well established principles showing a relation between exposure and allergic effect. Data should be obtained for more than one patient in more than one independent centre. All employed test methods should be validated.
- 'Limited evidence' means that isolated cases of allergic reactions are found in validated tests.
- 'Inadequate evidence' results from inadequate test methods or not sufficiently documented tests.
- When many people have been exposed to the allergen for an extended time period and no effect is found, there is evidence that the substance is not a sensitizer.
- Data from bioinformatics and *in vitro* tests may be used as 'supportive evidence'.

The type of tests will be different for contact sensitisation and airway sensitisation.

Once a protein is defined as an allergen (the first group) a classification according to type of allergen/protein may include:

1) Prevalence of the corresponding IgE in the allergic population: 'major' allergens (to which >50% of allergic patients react based on tests for IgE-binding potency) vs. minor allergens;
2) Prevalence in a certain geographic location (*e.g.* composition of local flora: birch pollen vs. olive pollen);
3) Severity of adverse reactions (*e.g.* potential to anaphylaxis);
4) Potential for cross-reactivity;
5) For modified proteins: relative IgE reactivity and T cell reactivity and their potential to induce the formation of protective antibodies.

Examples are provided in Chapter 6 on case studies. For the second group not enough data may be available to perform the second level grouping. The second level grouping will likely not influence the determination of the product as hazardous, but may provide additional insights on additional safety measures (*e.g.* availability of an emergency treatment in case of possible severe adverse reaction).

4.3.2 Activities leading to exposure

4.3.2.1 Laboratories

Routine laboratory handlings may lead to skin and/or mucus contact. Lab workers may come into contact with the recombinant allergenic protein or peptides during:

- handling cell cultures,
- collecting/harvesting cells and tissues,
- protein extraction,
- protein purification,
- protein evaluation procedures.

Various expression systems in bacteria (*e.g. E. coli*), yeast (with proteins excreted or non-excreted), virus systems (*e.g.* baculovirus, excreted or non-excreted proteins), animal cells and plants are applied. Exposure is possible whenever GM material is open-handled. Changing culture medium, making dilutions, and subculturing may be of risk when not properly performed and in case the protein is excreted. Harvesting techniques include grinding tissues, cell lysis, centrifugation, resuspension etc. and may lead to contact exposure. Proteins are extracted using various extraction techniques.

Due to their biological containment these expression systems in most cases do not present a risk of exposure to the environment (outside the contained facility).

Conventional protein purification may consist of ion exchange chromatography, gel filtration or affinity chromatography (Wallner *et al.,* 2004). For easy purification, affinity tags are used such as polyhistidyl or hexahistidyl tags or a FLAG tag that are fused with the coding sequence of interest.

It should be noted that once the GM allergen producing cells are no longer viable, purification and characterisation steps are no longer considered activities with GMOs. Strictly speaking they do not fall under the CU regulations, but are still covered by the workers protection legislation.

Aerosol formation is the main source for potential allergens to be inhaled. Typically pipetting, pouring of liquids, opening wet caps, using hot inoculation loops, centrifugation of open tubes, resuspending, mixing (vortexing) and cell-sorting produce aerosols. Further guidance on activities during which

aerosols can be created can be found in an FAQ document[24] of the Ministry of Infrastructure and the Environment.

4.3.2.2 Animal housing

Working with animals entails additional challenges. To test the recombinant allergens animal models (*e.g.* mice and guinea pigs) may be used. The animals are deliberately exposed to the protein via inhalation, tracheal or nasal intubation or intraperitoneal injections. Each of these treatments requires skilful personnel that master the distinct techniques. In general, applying anaesthetics, surgery and necropsy are additional manipulations at risk.

The animals may disperse allergens via wound leakage, coughing, sneezing, possibly also via excretions (saliva, urine and faeces) depending on the application method and type of protein.

Moreover, the animals may bite and scratch the animal caretakers and investigators. Skin scratches make it possible for large molecules to be taken up by the damaged cells. In case the recombinant proteins are present in the animal's saliva, the injured person is at risk.

Also dust may act as a carrier of antigens. Animal excretions (urine, faeces) may be dispersed as dust particles, as a result of movements by the animals (large animals, open cages) or when changing bedding material of (closed) cages.

Again, these points of attention are also valid for any newly produced protein. Once more, on the legal level, a distinction should be made between activities with and without GMOs.

4.3.2.3 Growth chambers and greenhouses

Plants may be genetically modified to produce allergens intentionally or unintentionally.

After the *in vitro* phase GM plants are typically grown in contained facilities such as growth rooms and greenhouses. Depending on the type and location of expression in the plant, employees may come in contact by touching stem, leaves, flowers and seed, or sap of damaged plant tissue. If expressed in the pollen cell wall, inhalation of pollen may be a threat. This may be prevented by bagging inflorescences before flowering. However, at seed harvest, pollen grains may still be present.

4.3.2.4 Large-scale production facilities

Production facilities with fermenters of more than 100 litres may produce a large variety of substances.

The large-scale production of allergens for diagnostic purposes is a realistic example of an industrial process with a risk for occupational allergies.

Industrial proteins like proteases and lipases (amylases and cellulases) for washing-powders, laccases for bleaching in the paper industry and enzymes used in tanning often are produced using GMOs. Likewise, enzymes for the food industry such as amylases, xylanase, pectinase and chymosine, and phytase and β-glucanase applied as feed additives, have allergenic characteristics. All may cause IgE-mediated allergy (Baur, 2005; Baur *et al.*, 2002; Belleri *et al.*, 2002; Green &

[24] http://www.ggo-vergunningverlening.nl/Veel_gestelde_vragen/Veel_gestelde_vragen_Ingeperkt_gebruik/Wat_wordt_verstaan_onder_aërosolvormende_handelingen

Beezhold, 2011; Smith *et al.*, 1997; Tarvainen *et al.*, 1991; van Kampen *et al.*, 2013). These enzymes may be modified by oligonucleotide-directed mutagenesis and DNA shuffling to optimise enzyme performance. Still they are structurally related with the allergenic native enzymes. The modifications may lower the number of allergenic sites, but potentially increase allergenicity.

Activities of special interest in relation to exposure are:

- inoculation of the fermenter,
- adding material,
- sample taking,
- transfer of content,
- draining and cleaning.

Using closed systems with a minimum of manual handling may reduce the danger for exposure.

Allergens are produced at large scale in plants as well (*e.g.* ANGANY Genetics[25]).

4.3.3 Conclusions

- In defining a threshold level below which no adverse effect occurs, sensitisation, being a crucial step in allergy development, should be the measure, but often goes unnoticed.
- Threshold levels are variable among persons and furthermore depending on duration and route of exposure.
- The relation between dose and effect may not be linear, and presumably different dose-response relationships exist for sensitisation and elicitation.
- Since in challenge studies some patients already react to the lowest dose, NOAEL/threshold are difficult, if not impossible, to determine.
- For contact and airway allergens, instead of thresholds, a classification is proposed with classification criteria depending on the available evidence.
- Proteins may be grouped in analogy with chemical substances based on evidence from validated tests. The classification may be further refined according to additional knowledge. Building on the notion that a harmful substance is only harmful in certain circumstances, the exposure potential in the variety of activities with allergen producing GMOs should be taken into account.
- Skin/mucus contact and aerosol formation leading to inhalation are the main modes of exposure. Skin contact is less important when working with proteins, due to their size.
- Safe (microbiological) techniques or good (laboratory) practices may reduce the risk of exposure substantially.
- Activities with GMOs that are no longer viable or with purified product, are not considered activities with GMOs. Strictly speaking they do not fall under the CU regulations, but are still covered by the workers protection legislation.
- Classification of activities will take into account the exposure potential, combining factors like route of exposure, amount/concentration and duration. Containment measures may be adapted for each of the consecutive steps in an activity or process. As is further exemplified in the case studies (Chapter 6), this means that the same allergen-producing organism may be handled with different containment measures depending to the activity. In this way ‘working with allergen producing GMOs’ may be subdivided in several activities each with appropriate containment measures.

[25] www.anganygenetics.com

4.4 Risk analysis

Whether GMO activities actually pose a risk to the worker is dependent on the probability of exposure and the allergenic properties of the newly produced protein. When the protein is unlikely to be an allergen or is not expressed (*e.g.* cloning activities, non-induced promoter), the risk is evidently low. Even if expressed in a cDNA bank only a small number of colonies will contain the allergen cDNA. Consequently, the amount of protein will be very low. Moreover, when not excreted, the probability of exposure is again very low. Also, recombinant allergens may be modified to only induce an immunologic reaction, not an allergic response. In these cases the risk may be reduced as well.

For activities where known allergens are expressed, the focus is on the possibility of exposure. The predominant route for exposure to protein allergens is inhalation of aerosols or dust particles. Since protein allergens are rather large molecules, skin contact is of minor importance except for persons with a weakened skin barrier (because of disease or injury). Also, allergens in a laboratory setting are usually present in a solution, *e.g.* during GMO culture and purification steps. Safe working practices will reduce exposure to a minimum. Workers will be exposed to the proteins only in case of a spill and/or when aerosols are produced, *e.g.* outside a class II MSC, due to careless manipulations. Correct actions after a spill, *e.g.* allowing aerosols to settle, immediately rinsing and washing of the exposed skin etc. will limit the risk.

Probability of exposure needs to be put in perspective. In the case allergenic proteins are manipulated as dry substances (*e.g.* pollen to extract DNA, RNA or proteins, freeze dried purified allergens etc.) the probability of inhaling the allergens is high. The duration of exposure in laboratories is generally speaking limited as compared with laboratory animal caretakers. The latter are exposed to the natural airborne dander allergens for extensive periods of time. In these settings occupational asthma is not uncommon (Seward, 2001).

Another important aspect to consider is the observation that the response to an allergen may be changed depending on the mode and site of exposure, the presence of adjuvants etc., as is experienced in allergen-specific immunotherapy (Linhart & Valenta, 2005). Nonetheless, due to the probable very low threshold amounts for allergens to induce adverse effects, any real exposure might be a risk.

The most important risk factor remains the condition of the individual that will perform the activity. Usually at the time of employment (*i.e.* adults) it is already known whether allergic reactions to some substances may be experienced or not (Valenta & Kraft, 2002). However, allergic disease development is a complex process and generally has two phases – sensitisation and elicitation – where the first phase may go unnoticed. Even if allergic persons are advised not to take part in the activities or take extra safety measures, careful consideration of the risk for all other employees is a necessity.

Taking all these elements into account leads to the assessment of the final (residual) risk that needs to be managed. When the risk assessment identifies unacceptable residual risks, further measures are needed to ensure safe operations.

5 Risk Management Measures

5.1 Standard Containment Requirements

Of the general measures applied at all levels of GMO containment to protect employees and the environment, the safe working practices are the basis ('*Regeling*'[26]). They include that the working environment is kept clean and orderly, and that eating, drinking, smoking, mouth pipetting etc. is prohibited to protect from skin contact with or ingestion of allergens. Also, hands must be regularly washed. Specific techniques prevent that aerosols are produced. Benches and equipment are decontaminated after use. Waste is decontaminated and protective clothing is kept at the working place. These general measures, also known as Good Microbiological Techniques (GMT), are described in guidance documents such as the WHO Lab Biosafety Manual, part IV (WHO, 2004) and they apply to all GMOs regardless of their allergenic potential and.

On top of these requirements for BSL I, access to a BSL II laboratory is restricted to authorised persons only (Table 4). All aerosol-producing activities with hazardous organisms are performed in a class II microbiological safety cabinet (MSC). Protective gowns are first decontaminated before washing. In BSL II laboratories animals are always handled with gloves (Annex 4, §4.1.1.3.2.w).

Access to plant growth rooms, greenhouses and animal housing is always restricted. Dispersal of GM pollen, seeds and any other reproductive organs (*e.g.* tubers) is prevented. Plants or animals in association with GMMs are subject to additional measures.

By large scale facilities for CU usually bioreactors with a volume of more than 100 L are targeted. For level II facilities emphasis is put on the prevention of aerosol formation and contamination of external surfaces during reactor manipulations.

All employees are instructed on safe working practices, the use of personal protective equipment (PPE), working procedures specific for the facility and steps to be taken by workers in the case of incidents and to prevent incidents.

The class II Microbiological Safety Cabinet

In a BSL II, the class II MSC is an essential piece of equipment that provides protection against aerosols produced during manipulations. Whenever a MSC is indicated in this document, reference is made to equipment fulfilling the conditions of EN12469:2000.

A class II MSC has a front aperture through which the operator can carry out manipulations inside the cabinet and is constructed so that he worker is protected, the risk of product and cross contamination is low and escape of airborne particulate contamination generated within the cabinet is controlled. While the European norm describes the performance criteria, there are different options to be considered. Some class II MSC are equipped with a second filter just after the work surface. In this way the interior part of the MSC remains clean, which makes such MSCs suitable to work with *e.g.* cytostatics. There are also different options in relation to recirculation of exhaust air. As aerosols with micro-organisms will be retained differently by the filter system than purified allergens (*e.g.* in powder), such options should be carefully considered on a case-by-case basis and may need to be justified in the risk assessment.

[26] Regeling genetisch gemodificeerde organismen van 28 mei 1998 (BWBR0009653, Stcrt. 1998, 108), Annex 4.

Table 4 **Main legally required containment requirements with an indication of protection against skin contact, inhalation, ingestion** (Between brackets the Dutch nomenclature is mentioned, as laid down in Annex 4 of the '*Regeling*', n.a.: not applicable)

Laboratories			
Measure	**Protecting**	**BSL I (ML-I)**	**BSL II (ML-II)**
Restricted access	Unauthorised persons in general	no	yes
Safe working practices	Skin contact, inhalation, ingestion	yes	yes
Lab coat	Skin contact	yes	yes
Class II MSC	Inhalation	no	yes
Waste decontamination	Employees and environment	yes	yes
Decontamination of lab coat before washing	Skin contact	when spilled	yes
Gloves when handling animals	Skin contact	n.a.	yes

Growth rooms and Greenhouses			
Measure	**Protecting**	**BSL I (PC-I, PK-I)**	**BSL II (PK-II)**
Restricted access	Unauthorised persons	yes	yes
Access via antechamber	Environment	no	yes
Insect screen	Environment	optional	yes
Safe working practices	Skin contact, inhalation, ingestion	yes	yes
Containment of reproductive parts	Environment Skin contact, inhalation	yes	yes
Protective clothing	Skin contact	no	no
Decontamination of waste containing reproductive parts	Environment	yes	yes

Growth rooms and Greenhouses for plants with GMMs			
Measure	**Protecting**	**BSL I (PCM-I, PKM-I)**	**BSL II (PCM-II, PKM-II)**
Restricted access	Unauthorised persons	yes	yes
Insect screen	Environment	no	yes
Safe working practices	Skin contact, inhalation, ingestion	yes	yes
Protective clothing	Skin contact	yes	yes
Gloves	Skin contact	no	no
Class II MSC	Inhalation	no	yes
Decontamination of waste	Environment	yes	yes

Animal housing			
Measure	**Protecting**	**BSL I (D-I)**	**BSL II (D- I[27])**
Restricted access	Unauthorised persons	yes	yes
Prevention of escape	Environment	yes	yes
Safe working practices	Skin contact, inhalation, ingestion	yes	yes
Protective clothing	Skin contact	no	optional
Gloves	Skin contact	no	optional
Class II MSC	Inhalation	no	optional
Decontamination of working clothes before washing	Skin contact	when spilled	when spilled
Decontamination of waste	Employees and environment	yes	yes

[27] If an animal produces harmful products, appropriate measures must be taken in the D-I environment.

Animal housing for animals with GMMs			
Measure	**Protecting**	**BSL I (DM-I)**	**BSL II (DM-II)**
Restricted access	Unauthorised persons	yes	yes
Prevention of escape	Environment	yes	yes
Safe working practices	Skin contact, inhalation, ingestion	yes	yes
Protective clothing	Skin contact	yes	yes
Gloves	Skin contact	no	no
Class II MSC	Inhalation	no	yes
Decontamination of working clothes before washing	Skin contact	when spilled	yes
Decontamination of waste	Employees and environment	yes	yes

Large scale facilities (*e.g.* bioreactors >100 L**)**			
Measure	**Protecting**	**BSL I (MI-I)**	**BSL II (MI-II, MI-III)**
Restricted access	Unauthorised persons	no	yes
Safe working practices	Skin contact, inhalation, ingestion	yes	yes
Protective clothing	Skin contact	yes	yes
Decontamination of working clothes before washing	Skin contact	no	yes

5.2 Specific Containment Requirements

On top of the standard containment measures to prevent GMOs from uncontrolled dissemination, exposure preventive measures are taken, especially when (putative) allergenic proteins are involved.

Contact may be avoided using PPE such a lab coat, (long) gloves, eye and face protection, and respiratory masks. It must be stressed that their effectiveness is often dependent on the user. Therefore, proper training should accompany any requirement for additional protective equipment. Care must be taken when choosing gloves: with latex gloves there is a risk of latex allergy. Moreover, wearing respirators, in animal housing for example, may be very uncomfortable when used during long periods. The risk analysis and management should take these elements into account.

Activities with a potential for aerosol formation should be carried out in a class II MSC. Furthermore, only closed centrifuge tubes should be used, etc. In animal housing exposure to dust may be reduced using facemasks, PAPR (Powered Air Purified Respirator), changing station for bedding material with air evacuation, etc. Protective clothing should in any case stay within the working environment. Attention should be paid to pack clothes carefully for transfer to the laundry to prevent others to become exposed.

Following a monitoring program in an animal facility, the amount of airborne particles was significantly reduced depending on the type of changing stations for individually ventilated cages (IVCs) compared to on table changing (Vangeel & De Vroey, 2012). To further reduce exposure using hoods, disposable P2 masks were recommended. No extra PPE was judged to be necessary in a room with roof air extraction.

However, in a literature study Vandenplas (2011) found that patients with occupational asthma using respiratory protective devices to reduce exposure on the long term experienced worsening of the disease compared to complete avoidance, although on the short term symptoms lessened. The author concludes that only reducing the exposure cannot be routinely recommended as a safe treatment strategy for already sensitised persons.

5.3 Medical screening

Since the prevalence of allergies is highly dependent on a person's predisposition, workers may be screened before starting activities with allergens. A personal history of atopy will increase the probability of allergy development to the substances and organisms one is working with. Yet, atopy screening results are not sufficiently predictive for future occupational sensitisation (Wilken *et al.*, 2012). Atopic persons are advised, depending on the severity of their symptoms, to completely avoid the substances they are susceptible to.

Predisposition tests that can be used in a pre-placement medical examination are the classical skin prick tests, IgE serology (type I allergy) and patch test (type IV allergy). Specific sensitisation for the work-associated allergens can be detected using these tests. It is, however, acknowledged that the value of all of these approaches to predict disease development is limited and false positives do occur (Seward, 2001). Therefore, this screening may be used as an indication only, *i.e.* to identify those workers at a higher risk of developing work-related allergies, not as an exclusion criterion (Wilken *et al.*, 2012).

Several commercial tests are available for the detection of serum specific IgE antibodies (Lucassen *et al.*, 2010), such as the Allergy Lateral Flow Assay (ALFA[28]), the 'ALLERG-O-LIQ System[29]', 'ImmunoCAP[30]' and the ISAC® test.

Medical surveillance for predisposition or for serum specific IgE antibodies may be part of an occupational health and safety program (as required by the workers protection legislation). It would make an early diagnosis possible and immediate appropriate interventions to further prevent adverse effects or progression of the disease. Serum banking might be useful to facilitate clinical diagnosis and/or treatment in the event of an occupational exposure. It establishes a baseline against which future changes in health status can be measured. Medical surveillance may include periodic examinations and questionnaires. However, when working with common allergens like tree or grass pollen allergens, it may be difficult to discriminate between a lab-acquired allergy or allergy developed from exposure outside.

Additionally, employees may be educated about medical risks, including recognizing the symptoms and signs of allergy.

In a high risk environment it is recommended to have a first aid/rescue kit available to assist individuals with anaphylactic shock. While the exact content needs to be established with the occupational physician, it will likely contain epinephrine that counteracts the effects of allergic mediators on smooth muscle and vasculature. Epinephrine autoinjectors are available under the trade names Anapen®, Epipen® and Jext®. Everyone involved in the activity should be trained to recognise the symptoms and to immediately response. In case allergic persons choose to take part in the activities, although advised against, they are advised to carry such autoinjectors with them. Making available autoinjectors should be accompanied by proper training on recognizing the symptoms and on proper use of the devices.

5.4 COGEM advisory reports

COGEM advised so far on several applications for working with allergen producing GMOs in CU (CGM/931020-33, CGM/981008-06, CGM/990311-06 and CGM/990419-04). Examples of standard

[28] http://www.montwell.com.tr/Download/drfooke/drfooke-16.pdf
[29] http://www.montwell.com.tr/Download/drfooke/drfooke-17.pdf
[30] http://www.questdiagnostics.com/home/physicians/testing-services/by-test-name/immunocap

classification by the GMO office are IG 02-063/02; IG 03-188/03 and IG 12-109/00. The activities in those cases concerned cloning of known allergen genes in *E. coli* K12 and yeast, and transfection to animal cells. In conformity with Annex 5, §5.2.f of the '*Regeling*', they were advised to be performed at BSL II. The obligation to wear gloves was added as an extra safety measure.

COGEM reduced in one case (CGM/981008-06) the containment level requirement from BSL II to BSL I after a positive advice of an allergist (CGM/990419-04).
The Provisional Commission Genetic Modification (the forerunner of COGEM) advised in CGM/931020-33 to clone Der p 2 in *E. coli* K12 and *S. cerevisiae* at BSL I. At that time a previous legislation was in vigour.

In recommending containment measures one option is to classify activities with known allergens as BSL II activities. This option would solve the risk of respiratory exposure, since a class II MSC is required for aerosol generating activities. But even then standard measures do not prevent skin contact and gloves are most often advised as an extra precaution. Another option may be to perform the activities at BSL I with extra precautions to prevent inhalation (class II MSC/ PAPR or similar) and skin contact (gloves). However, in this case unrestricted access to the containment area may be an issue.

5.4.1 Conclusions

- Safe working practices are the basis to protect employees and the environment in CU conditions.
- Standard containment measures of BSL I are not sufficient to protect the workers from exposure to allergens.
- BSL II requirements provide protection for inhalation (class II MSC) but need an extra safety measure to protect from contact (gloves).
- Predisposition tests have limited value to predict disease development.
- Medical surveillance may be part of an occupational health and safety program.
- Creating awareness among workers and education about risks and symptoms is a valuable tool.

6 Case studies

This chapter describes some possible scenarios of research activities or production processes. The cases are described in general terms indicating elements that are relevant for the RA without going into technical details. The vector constructs are supposed to be listed in Annex 2.1.1 and 2.1.2 of the '*Regeling*'[31]. While the types of activities are assembled from several scientific publications to give a realistic picture of possible scenarios, the subsequent RA is performed by the authors of this report. Likewise, the risk management measures that follow from the risk level are the authors' proposals. In preparing this report experts in the field were consulted on the topic. Their comments are integrated in these case studies.

Only the RA concerning the allergen is described. There may be other concerns related to the GMO and/or the activity, that should be addressed in the RA and that may require additional containment measures. *E.g.* the receptor/host micro-organisms in the examples below all belong to pathogenicity class 1 (PG 1) (microorganisms that are unlikely to cause human disease or animal disease). In case receptor organisms of a higher pathogenicity class are used, additional precautions may be warranted. Also, the spread of GMOs beyond containment should be prevented. In consequence, supplementary containment measures may be required. As these are not included here, the complete set of containment measures may be more comprehensive and result in a higher containment level than inspired purely due to the allergenic potential.

The first steps in conducting a risk analysis are the listing of properties of the allergen and the harm it can cause, followed by the activities potentially leading to exposure. Subsequently the risk is analysed and is presented in table format. The proposed allergen related management measures are for healthy employees, without known allergies.

For each case study a table is provided presenting consecutive activities and indicating for each step whether the protein is expressed and/or present. The next column indicates whether exposure is expected. The combination of expression/presence and exposure results in the corresponding risk of that activity. The 'Management' column sets safety measures as proposed by the authors, followed by a column indicating the containment level that best fits the management proposal, possibly with additional measures. Activities shaded in grey refer to activities not falling under the GMO legislation. In that case no containment level is indicated, but safe-working practices such as GMT and quality systems such as GLP may be implemented, sometimes further improving safety by the use of additional equipment and/or PPE.

6.1 Examples of basic research on allergens

6.1.1 Working with wild-type and modified Bet v 1 from birch pollen (*Betula verrucosa*) in *Escherichia coli* K12

Description

cDNA inserts are PCR amplified and introduced in plasmids for expression in *E. coli* K12 (PG 1). Both non-fusion and fusion (*e.g.* with poly-His tag) versions are made. In this system the lacUV5 promoter leads to inducible production of the recombinant protein. When fusions are envisaged a cleavage site for tobacco etch viral protease is present in the vector to remove the His tag after protein purification. Site-directed mutagenesis of Bet v 1 made a mutant carrying 6 amino acid exchanges, resulting in very low IgE binding without changing the T cell activation capacity.

[31] http://www.ggo-vergunningverlening.nl/Vergunningverlening/Documenten

E. coli cells are transformed with the engineered plasmids, selected and grown in liquid medium. Protein expression is induced by the addition of IPTG. After expression, cells are harvested by centrifugation, and pellets are resuspended. The cells are then disrupted by freezing in liquid nitrogen followed by thawing at 37°C. This step is repeated twice. Proteins are recovered in the supernatant or in the insoluble protein fraction (depending on the Bet v 1 isoform) after centrifugation.

Characteristics of the allergen

- Based on the classification proposed in this report, this protein will be considered a group 1 protein (well documented allergen);
- Pollen protein found in birch, function unknown (pathogenesis-related protein, PR-10) (IUIS[32]);
- Major allergen (>95% of birch pollen-allergic patients showing IgE reactivity to Bet v 1) (IUIS; Valenta *et al*., 1991);
- Induces IgE cross-reactivity with many pollen and food proteins (panallergen) (Hauser *et al.,* 2010; Weber, 2001);
- Heat-labile;
- Induces allergic rhinitis (seasonal discomfort).

Planned activities

- Cloning;
- Production in *E. coli*;
- *E. coli* cell harvest.

Risk analysis

Table 5 reviews the subsequent steps in the process, analysing potential exposure and risk for the laboratory worker. The allergen is produced only upon addition of the inducer, IPTG. The preceding steps therefore do not pose any risk of contact or inhalation. Since the expression construct has no excretion signal the protein will stay inside the bacterial cells. Only when cells disintegrate they end up in the culture medium. Consequently, only in case of a spill (dropping flasks) employees are exposed, but to very small amounts of allergen.

Minute exposure is also possible at harvest when not handling according to safe practices (gently pouring out fluids, allowing aerosols to settle after centrifugation before opening tubes). Only after cells are broken, proteins are set free in the liquid fraction in high amounts. Careful operations will reduce aerosol formation. When spilled, contact may occur via skin and inhalation of small droplets. Skin contact will not represent a risk, especially when the skin is intact and when rinsed immediately, as required, and because Bet v 1 acts predominantly via airway contact.

Although all handlings and exposure potential are the same, the risk is here judged to be lower for the recombinant Bet v 1 due to its reduced capacity to evoke an IgE reaction (the hazard element of the equation is low). Though, experts may disagree with this reasoning. Also, in this example the characteristics of the modified gene product are known. In practice, in an experimental setting one may aim at creating hypoallergens, but this may only become proven afterwards. Until then, the modified molecules should be handled the same way as the wild-type allergen (worst-case scenario).

[32] http://www.allergen.org/viewallergen.php?aid=129

Table 5 Wild-type and modified Bet v 1 in *E. coli* K12

Allergen	Activity	Expression/ Presence of allergen	Exposure to allergen	Risk	Management	Containment level
Wild-type Bet v 1 in expression vector with inducible promoter	Plasmid construction	None	None	None	None	
	Transformation to *E. coli* for production	Negligible	None	Negligible	None	BSL I
	Production in *E. coli*, promoter induction	Inside *E. coli* cells	Minute exposure via skin or inhalation (spills)	Very low	Safe practices	BSL I
	Harvest cells via centrifugation	Inside *E. coli* cells	Minute exposure when pouring and opening tubes	Very low	Safe practices	BSL I
	Resuspension of cell pellet	Inside *E. coli* cells and in buffer from broken cells	Exposure from aerosol formation, unless performed in closed vials	Low	Open vials: handle in class II MSC, gloves	BSL I (closed vials) or BSL II + gloves
	Cell disruption (freeze/thaw)	In liquid and/or debris (closed vials)	via rupture of vials	Medium	Class II MSC, gloves	BSL II + gloves
	Centrifugation of disrupted cells	In supernatant and/or debris	Exposure when pouring and opening tubes	Medium	Manipulations in Class II MSC; gloves; centrifugation in closed recipients	
Bet v 1 with reduced IgE reactivity in expression vector with inducible promoter	Plasmid construction	None	None	None	None	
	Transformation to *E. coli* for production	Negligible	None	Negligible	None	BSL I
	Production in *E. coli*	Inside *E. coli* cells	Minute exposure via skin or inhalation (spills)	Very low	Safe practices	BSL I
	Harvest cells via centrifugation	Inside *E. coli* cells	Minute exposure when pouring and opening tubes	Very low	Safe practices	BSL I
	Resuspension of cell pellet	Inside *E. coli* cells and in buffer from broken cells	Exposure from aerosol formation	Very low	Safe practices	BSL I
	Cell disruption (freeze/thaw)	In liquid and/or debris (closed vials)	Rupture of vials	Very low	Face mask (to protect from rupturing vials)	BSL I +face mask
	Centrifugation of disrupted cells	In supernatant and/or debris	Exposure when pouring and opening tubes	Very low	Safe practices	

6.1.2 Working with native and modified Der p 1 from house dust mite (*Dermatophagoides pteronyssinus*)

Description

The aim is to produce the whole genomic sequence encoding a precursor protein ProDer p 1 including a signal peptide, and a modified form in insect cells (*Drosophila*), yeast cells (*Pichia pastoris*) (PG 1) or Chinese hamster ovary (CHO) cells. The precursor protein is enzymatically inactive (no protease activity).

Genomic DNA is isolated from a whole mite culture. Using PCR the Der p 1 gene is amplified and subcloned in pUC19 in *E. coli* K12. Alternatively, cDNA cloning is performed by PCR using mRNA purified from house dust mite. The modified versions are codon optimised for higher expression in their eukaryotic expression cell systems. Via an intermediate vector, the distinct sequences are cloned in expression vectors adapted for each of the three expression systems.

The vector for *Drosophila* has an inducible expression cassette (*Drosophila* metallothionein promoter). Together with the coding sequence a secretion signal peptide sequence from baculovirus is integrated. Schneider 2 cells (derived from late embryonic stages of *Drosophila melanogaster*) are transfected with the recombinant plasmids. Transformants are selected on antibiotic containing medium. To induce expression $CuSO_4$ was added to the medium. After 3 days the culture is centrifuged and the supernatant collected for purification.

The yeast expression vector includes the *Saccharomyces cerevisiae* alpha-factor promoter to express the inserted gene and to efficiently secrete the corresponding protein into the culture medium. *P. pastoris* is transfected and selected on antibiotic containing agar-medium. Production of the allergen is performed growing the yeast in liquid medium. Cells are then precipitated by centrifugation and the supernatant is further used.

CHO cells are transfected with a constitutive, high-level expression mammalian vector containing the CMV promoter and N-terminal His tag for easy purification. The CMV promoter is known to have a cryptic *E. coli* promoter. Spent culture media is harvested after 10 days of culture, by centrifugation and stored for further purification.

The protein in the supernatant may be purified first by enriching over a sepharose column, ultrafiltration and by gel filtration chromatography.

Further analysis of all purified versions of allergen may be done by enzymatic assays, SDS-PAGE, Western blot analysis, ELISA, serum binding tests, or using *in vitro* cell systems such as basophils for testing histamine release, etc.

Characteristics of the allergen

- Based on the classification proposed in this report, this protein will be considered a group 1 protein (well documented allergen);
- Cysteine protease; a 24 kDa protein located in the mite gut and becoming airborne after excretion in faecal pellets (IUIS[33]);
- Major allergen (the prevalence of anti-Der p 1 IgE antibodies in patients with mite allergy is higher than 80%) (UniProt[34]);

[33] http://www.allergen.org/viewallergen.php?aid=289
[34] http://www.uniprot.org/uniprot/P08176

- Able to decrease the barrier function of the epithelial cell layers due to protease activity (Kauffman *et al*., 2006);
- Heat-labile;
- Inducing strong reactions in skin and airway mucosa from atopic subjects resulting in atopic dermatitis as well as allergic rhinitis and asthma.

Planned activities

- Cloning;
- Transfer to production cell system;
- Culturing production cells *in vitro*;
- Purification;
- Testing in *in vitro* cell systems.

Risk analysis

Table 6 summarises the subsequent steps in the process, analysing potential exposure to the proteins and risk for the laboratory worker. The distinction between the RA for wild-type and codon optimised Der p 1 is the amount of protein that is produced by the different cells (*e.g.* 5 to 10-fold increase). This element is not further elaborated in the table as some activities with wild-type Der p 1 already require the use of a class II MSC.

Although not a GMO activity, the first challenge in terms of allergen exposure is the isolation of DNA or RNA from a whole mite culture (*e.g.* 1 g may contain 15,000 mites equal to 300 µg of Der p 1). Cloning and vector construction do not pose a high risk using the eukaryotic expression vectors with inducible promoters, as there is no expression. The construct destined for CHO transfection has the CMV promoter that might leak in *E. coli*. This has to be taken into account in the RA or otherwise the problem may be solved by inserting a prokaryotic transcription terminator between the CMV promoter and the gene of interest.

In all three production systems the allergen is excreted in the culture medium. Again spilling accidents or careless handling of the cultures may result in skin contact or inhalation of aerosols. In this example the precursor protein has no protease activity. Consequently the damaging effect on skin and airway mucosa is absent (autocatalysis to form the mature allergen does not take place during expression and purification steps). As in the previous example the harvest step is crucial.

In the subsequent purification steps the points of attention are again correct handling of liquids. The same is true for all kind of assays. The highest potential for inhalation of allergens occurs whenever allergens are handled as a powder. In this example the allergen is freeze-dried and stored for further use later on. However, all these steps are no longer regarded as activities with GMOs.

Overall, the amount of allergens lab workers are potentially exposed to during recombinant DNA activities is rather limited compared to collecting of and isolation from natural allergen sources (such as pollen) or compared to the purification of the produced proteins where the concentration is gradually increasing. Especially dry formulations are of specific concern as concentrated powder can easily spread.

Table 6 Wild-type and modified ProDer p 1 in eukaryotic expression systems

Allergen	Activity	Expression/ Presence of allergen	Exposure to allergen	Risk	Management	Containment level
Wild-type and optimised ProDer p 1 in expression vector with inducible promoter	Genomic or cDNA cloning in *E. coli*	Inside *E. coli* cells, but negligible	Minute exposure via skin or inhalation (spills)	Very low	Safe practices	BSL I
	Plasmid construction (subcloning)	None	None	None	None	BSL I
	Transfecting *Drosophila* or yeast cells	None	None	None	None	BSL I
	Production in *Drosophila* or yeast cells	In culture medium	Via skin or inhalation (spills)	Low	Safe practices	BSL I
	Harvest cells via centrifugation	In supernatant	When pouring and opening tubes	Medium	Manipulations in Class II MSC; gloves; entrifugation in closed recipients	BSL II + gloves
Wild-type and optimised ProDer p 1 in expression vector with CMV promoter	Synthetic DNA cloning in *E. coli*	Inside *E. coli* cells	Minute exposure via skin or inhalation (spills)	Very low	Safe practices	BSL I
	Transfecting CHO cells	None	None	None	None	BSL I
	Production in CHO cells	In culture medium	Via skin or inhalation (spills)	Low	Safe practices	BSL I
	Harvest cells via centrifugation	In supernatant	When pouring and opening tubes	Medium	Class II MSC, gloves	BSL II + gloves
Protein purification	Columns, filtration	In liquid	When pouring, pipetting	Medium	Open manipulations in class II MSC, gloves; possibly filter on freeze dry exhaust	
	Freeze drying	In powder/liquid	Inhalation of dust	High		
(*In vitro*) assays	Preparing test solution	In powder/liquid	Inhalation of dust	High		
	Performing tests	In liquid	When pipetting	Medium		

6.1.3 Experiments with wild-type and modified grass pollen allergen (*Phleum pratense*) in animals

Description

The research aims to test the vaccination capacity of a recombinant, hypoallergenic Phl p 7 protein (group 7 *Phleum pratense* allergen). Next to protein vaccination, also DNA vaccination is investigated. A mouse model will be used to test the effect of vaccination against sensitisation in an immunoprophylactic experimental setup. Female Balb/c mice (6 weeks old) were immunised via intraperitoneal injection 3 times at 2-week intervals using Phl p 7-DNA-construct or purified recombinant Phl p 7 adjuvanted with aluminium hydroxide (ratio allergen/adjuvant 1/20). As a control, 1 group of mice was immunised with saline alone. The mice were subsequently sensitised with adjuvanted, purified, natural Phl p 7, again via intraperitoneal injection (3x weekly).

For the actual test mice were airway challenged to an aerosol containing the natural Phl p 7. To prepare the inoculum purified natural Phl p 7 is resuspended in inoculation solution. Mice are anaesthetised and 50µl of the allergen suspension is administered to the lungs using a syringe without needle. To have a homogeneous distribution 0.5ml of air is administered twice. The treated animals are returned to their IVCs while still sleeping. This treatment is repeated 4 times at daily intervals. Two weeks after the last challenge the animals are euthanized. For each mouse, serum, bronchoalveolar lavage fluid (BALF), and spleen are collected for further analysis.

Characteristics of the allergen

- Based on the classification proposed in this report, this protein will be considered a group 1 protein (well documented allergen);
- Calcium binding protein (polcalcin family) (IUIS[35]);
- Minor allergen (<10% of grass pollen-allergic persons showing IgE reactivity) (IUIS);
- Strong IgE reactivity;
- Cross-reactive with other pollen polcalcins (panallergen) (Hauser *et al.,* 2010);
- May induce asthma, rhinitis and conjunctivitis (seasonal discomfort).

Planned activities

- Immunisation: administration of hypoallergenic Phl p 7 via injection;
- Immunisation: administration of Phl p 7-DNA-construct via injection;
- Sensitisation: administration of natural Phl p 7 via injection;
- Challenge: administration of natural Phl p 7 + air;
- Euthanasia and dissection.

The endotoxin-free plasmid has been prepared elsewhere.

Risk analysis

In this example only the experimental steps with plasmid DNA in conjunction with animals are considered GMO activities. The underlying reasoning is the possibility, however small, to form GMOs by recombination or uptake and integration of DNA in cells. In this case mice as well as laboratory workers (needle stick injury) are at risk.

The described activities where only purified recombinant and natural Phl p 7 are used are strictly speaking no GMO activities. Nevertheless, exposure may present a hazard to the employees. As in the previous example working with the allergen in dry phase (powder to be dissolved) has the highest risk, due to inhalation of dust particles (Table 7). However, the risk in case of the hypoallergenic form may be very low. Once in solution/suspension contact potentially may be harmful when the skin is

[35] http://www.allergen.org/viewallergen.php?aid=511

injured. This may be the case with a needle stick accident. Adhering to safe practices will reduce the risk of injury.

In the challenge step the mice are anaesthetised to reduce stress and suffering from the administration onto the lungs. Nonetheless, it is still possible that liquid is spilled or aerosols formed due to coughing. The subsequent aeration of the mice, again, may induce aerosol production.

With DNA vaccinated mice these steps are performed at BSL I because of the potential presence of a GMO. For human safety a class II MSC and gloves are advised. When the animals are euthanized and dissected, the administered allergens are already cleared from the lungs (2 weeks later).

Table 7 Testing wild-type and modified Phl p 7 in animals

Allergen	Activity	Expression/ Presence of allergen	Exposure to allergen	Risk	Management	Containment level
Hypo-allergenic Phl p 7	Preparation of immunisation solution	In powder/liquid	Inhalation of dust/aerosol	Very low	Safe practices	
	Intraperitoneal injection	In liquid	Needle stick injury	Very low	Safe practices	
Wild-type Phl p 7 to challenge Hypo-allergenic Phl p 7 vaccinated animals	Preparation of sensitisation/ challenging solution	In powder/liquid	Inhalation of dust/aerosol	Medium	Class II MSC, gloves	
	Intraperitoneal injection (sensitisation)	In liquid	Needle stick injury	Medium	Safe practices	
	Injection of allergen in lungs (challenge)	In liquid	Via skin or inhalation	Medium	Class II MSC, gloves	
	Aeration	In aerosol	Inhalation	Medium	Class II MSC, gloves	
	Euthanasia, Dissection	None	None	None	None	
Phl p 7-DNA-construct	Preparation of immunisation solution	None	None	None	Safe practices	
	Intraperitoneal injection	None	Needle stick injury + subsequent expression	Very low	Safe practices	BSL I
Wild-type Phl p 7 to challenge Phl p 7- DNA vaccinated animals	Preparation of sensitisation/ challenging solution	In powder/liquid	Inhalation of dust/aerosol	Medium	Class II MSC, gloves	
	Intraperitoneal injection (sensitisation)	In liquid	Needle stick injury	Medium	Safe practices	BSL I
	Injection of allergen in lungs (challenge)	In liquid	Via skin or inhalation	Medium	Class II MSC, gloves	BSL I + Class II MSC, gloves
	Aeration	In aerosol	Inhalation	Medium	Class II MSC, gloves	BSL I + Class II MSC, gloves
	Euthanasia, Dissection	Negligible	Skin contact	Negligible	None	

6.2 Examples of allergen production

6.2.1 Large-scale production of wasp (*Vespula vulgaris*) Ves v 1 in yeast

Description
Recombinant Ves v 1 is produced in large-scale bioreactor (fed-batch). The gene was mutated by site-directed mutagenesis (for optimal production) and has been cloned behind an inducible promoter and secretion signal of the α-mating factor from *Saccharomyces cerevisiae*. The methylotrophic yeast *Pichia pastoris* is the host organism (PG 1). The vector used for transformation is stably integrated into the genome. Methanol serves as inducer and carbon source. The protein is excreted in the culture medium. Harvest and purification is performed using expanded bed chromatography in closed system: production is stopped heating the reactor to 68°C, the culture is diluted, pH-adjusted and run over a nickel-chelating affinity column (several steps combine ion exchange, cell separation, concentration and clarification).

Characteristics of the allergen
- Based on the classification proposed in this report, this protein will be considered a group 1 protein (well documented allergen);
- Phospholipase A1 (IUIS[36]);
- Major allergen;
- No cross-reactivity with homologues in other hymenoptera species (species specific) (Seismann *et al*., 2010);
- Heat-stable;
- May cause life-threatening IgE-mediated anaphylactic reactions in allergic individuals.

Planned activities
- Production
- Purification

Risk analysis
In this example the production strain has been prepared, selected for high yields and tested for impurities. The recombinant yeast inoculum is built up in several steps going from baffled shake flasks to small bioreactors before the production bioreactor is started. Due to the need for promoter induction, no Ves v 1 is produced in these stages (Table 8). Once the inducer is added the concentration of allergen in the culture medium will increase over time. The bioreactor is a closed system. Feeding and sampling during production is done using aseptic devices that reduce contact to a minimum. For harvest and purification the bioreactor content is pumped to the expanded bed chromatography device.

As threshold may be very low, minute amounts may cause adverse effects. This must be taken into account because at first sight the exposure of workers in this case may be low compared to *e.g.* workers in the bakery industry or industrial enzyme production facilities, where occupational allergy often arises. Still the continued year-round exposure to very low amounts may also induce a reaction.

Although the probability of exposure via leakage or spill might be low, the amount of allergen present in the culture medium is high and consequences may be severe. However, the usual exposure route is via the injured skin (sting). The risk is therefore judged to be low, provided that gloves are worn during

[36] http://www.allergen.org/viewallergen.php?aid=644

cleaning of a spill, sampling, connecting tubes, etc. A first aid/rescue kit should be available to assist individuals with anaphylactic shock in case of an accident.

These allergens are produced in a pharmaceutical environment, requiring adherence to strict regulations. Although it is acknowledged that Good Large Scale Practice (GLSP) regulations will only protect the product, the safety of employees is top priority as well. In general, contact with concentrated pure pharmaceuticals should be avoided any time.

Other concerns may require additional containment measures. In spite of the fact that the allergen production is controlled by an inducer, the potential impact of an uncontrolled dissemination beyond containment could have severe consequences. It is therefore likely that the risk assessors require more stringent measures (COGEM would probably propose MI III; Annex 5, §5.7.3a of the '*Regeling*') to avoid dispersal. For large-scale production units, COGEM always advises on specific containment measures, as no two production facilities are identical.

Table 8 Large-scale production of wasp Ves v 1 in *Pichia pastoris*

Allergen	Activity	Expression/ Presence of allergen	Exposure to allergen	Risk	Management	Containment level*
Ves v 1 in expression vector with inducible promoter	Inoculum development	None	None	None	None	BSL I
	Inoculation of fermenter	None	None	None	None	BSL I
	Addition of inducer	None	None	None	None	BSL I
	Production	In liquid	Skin contact (leakage of bioreactor)	Low	Adjust spill collection capacity, gloves, emergency kit	BSL I + gloves, emergency kit
	Sampling	In liquid	Skin contact (spill)	Low	Safe practices, closed system, gloves, emergency kit	BSL I + gloves, emergency kit
	Harvest purification	In liquid	Skin contact (leakage)	Low	Safe practices, adjust spill collection capacity, gloves, emergency kit	BSL I + gloves, emergency kit

* Indications strictly based on the risk posed by the allergen.

6.2.2 Production of apple Mal d 2 in tobacco plants

Description

The gene for Mal d 2, a thaumatin-like allergen of apple, was cloned in a tobacco mosaic virus (TMV) derived expression vector. Capped infectious viral RNA is generated *in vitro* from purified plasmid DNAs by using a T7 RNA polymerase. To infect *Nicotiana benthamiana* plants leaves are rubbed using carborundum powder to damage the surface and 2 µg of RNA is applied. The RNA virus replicates extrachromosomally and redirects protein synthesis of the host cells to express high levels of the protein of interest throughout the plant. The TMV-U1 coat protein promoter my produce allergen proteins for as much as 10% of the dry weight of an infected leaf. The protein is expressed transiently and leaves or complete plants are harvested 2-3 weeks later. Plant tissue is frozen in liquid nitrogen, ground to a fine powder, and extracted with buffer to obtain an antigen containing solution followed by a purification step.

Characteristics of the allergen

- Based on the classification proposed in this report, this protein will be considered a group 1 protein (well documented allergen);
- Thaumatin-like protein, group 5 pathogenesis-related (PR) proteins (antifungal) (IUIS[37]; Krebitz *et al*., 2003);
- Major apple allergen (75% of apple allergic patients' sera reacts with this allergen) (Krebitz *et al*., 2003);
- Homologous to Pru av 2 (*Prunus avium*, cherry) (Krebitz *et al*., 2003);
- Heat-labile;
- May cause a food allergy.

Planned activities

- Production
- Purification

Risk analysis

Although virus RNA is produced *in vitro*, this activity is designated a GMO activity. However, no allergen is produced yet (Table 9). Plants are inoculated in a contained greenhouse/growth chamber. Successfully infected plants will produce the allergen, complete with proper folding and post-translational modifications. When handling plants and especially during harvest, plants may be damaged and may leak cell content. Skin contact may become problematic only when injured. The same is true for subsequent steps in processing the plant tissue and allergen purification. In these last steps the protein may be present in a high concentration. Safe working practices prevent mouth contact (ingestion).

Plants in association with GMMs are subject to additional measures, such as wearing a lab coat and decontamination of all waste (Table 4). For the production of the allergen *per se* BSL I with mandatory use of gloves would be sufficient. However, the capped RNA virus is an infectious plant pathogen requiring BSL II.

[37] http://www.allergen.org/viewallergen.php?aid=426

Table 9 Large-scale production of apple Mal d 2 in *Nicotiana benthamiana*

Allergen	Activity	Expression/ Presence of allergen	Exposure to allergen	Risk	Management	Containment level
Mal d 2 in TMV expression vector	Production of viral RNA	None	None	-	None	BSL II
	Inoculation of plants	None	None	-	None	BSL II
	Growing inoculated plants	Inside plant cells	Skin contact when damaging tissue	Very low	Safe practices, gloves, lab coat	BSL II + gloves
	Harvest	Inside plant cells	Skin contact	Very low	Safe practices, gloves, lab coat	BSL II + gloves
	Freezing/ grinding/ extraction	In biomass/ extract	Skin contact	Very low	Safe practices, gloves, lab coat	BSL II + gloves

7 Conclusions

Over the last decades, the prevalence of allergic disease has been almost continuously increasing. Many previously unknown allergens have been identified. The growing awareness concerning allergic disease has led to a cautious risk classification of activities with GMOs expressing allergens in CU. Since recombinant allergens may, in contrast to their natural counterparts, be prepared with very high purity in high concentrations, caution with these products is warranted.

Risk classification depends on the characteristics of the allergen one is working with and the probability of being exposed to it. Whereas a catalogue of activities with potential exposure can easily be composed by carefully examining each step in a working procedure, the hazard component in the risk equation of a given allergen is hard to determine. The approach for dealing with allergens is less developed. This report provides elements to support the RA for activities with GMOs that produce allergens. In particular the feasibility for classification is investigated.

Unlike for other hazardous substances such as toxins, no clear-cut indication exists on the threshold below which no harm is expected. The person to handle the (potential) allergen is the first and most important factor of uncertainty. While most people tolerate these allergens, part of the population is sensitive in various degrees, depending on genetic background, age, immune status and environment. In consequence, no safe threshold can be established for a given allergen. Furthermore, allergy development involves a sensitisation step followed by elicitation. Cross-reactivity increases complexity.

Related to this, there is currently no established model to reliably predict the allergenicity of a molecule. While *in silico* studies may indicate the potential for cross-reactivity between allergens, they only allow for structural similarities to detect and do not take into account the route of exposure or the form in which the allergen is presented. Also *in vitro* and animal models have their shortcomings. Future developments in test methods may bring solutions and should be watched closely.

Keeping in mind that allergenic proteins have specific characteristics, a proposal is made to classify them based on guidelines for chemical substances that may cause sensitisation. Also, when determining intrinsic allergenicity of a recombinant protein - insofar this can be determined - any modification compared to the natural form has to be taken into account as well. Classification of allergens may further include elements such as prevalence of the corresponding IgE in the allergic population, occurrence in the environment, severity of disease, and cross-reactivity potential. On this basis, it is proposed to distinct four classes of proteins:

1) Known allergens (scientifically established evidence);
2) Probably allergenic proteins (some indications present);
3) Proteins for which allergenicity cannot be excluded (single cases, ill-defined studies);
4) Proteins with no indication of allergenicity.

Based on this classification, group 1 and 2 proteins would be considered hazardous substances, whereas group 4 proteins would not. For group 3 proteins additional evaluation or cautionary preventive measures would be warranted.

Since GMO legislation requires classifying activities with GMOs producing hazardous substances as BSL II (or higher) activities (Annex 5 to the '*Regeling*'), applications relating to known allergens most often are categorised as a whole as BSL II activities. However, some circumstances and types of molecules may allow for less stringent containment measures at certain steps.

The second element in the ERA is indeed the likelihood of the hazard to be realised. This is partly defined by the possibility of exposure and –again- the health status of the laboratory worker. Exposure is not only defined by the type of handlings, but also whether the protein is expressed, in which amounts and where (*e.g.* intracellular, in cell wall, or excreted). This may necessitate to divide activities into several steps, each resulting in a corresponding risk classification.

Although sensitization by low doses cannot be excluded, occupational allergies are often the result of exposure to high doses for longer periods, conditions that in general do not occur in research laboratory settings. In general the amounts one might be exposed to in CU conditions are rather low. Even in large-scale production facilities where relatively important quantities may be produced, exposure will be limited as the culture of a GMO already requires stringent containment measures, next to the strict manufacturing conditions for pharmaceuticals (diagnostics, vaccines).

The most prominent exposure routes are inhalation of aerosols and contact via skin or mucosa. While the basic set of good laboratory practices for dealing with GMOs at BSL I will already provide protection, additional protective measures may include the use of a class II MSC, specific respiratory protection and gloves. This can in part be achieved by requiring BSL II containment. For specific cases -to be determined case-by-case-, *e.g.* a group 3 protein, BSL I containment with additional precautions may provide a suitable alternative. This option may provide a bridge to other activities that are not subject to high containment requirements. Examples are establishing gene banks where expression is not expected, and working with organisms with endogenous allergens that are not excreted. This approach can be justified by the low or negligible probability of exposure and is in line with the general risk assessment approach of evaluating hazards in the context of the activity and the exposed environment.

While the information reviewed in this report can assist both risk assessors and developers in their RA for the CU of GMOs intended to express allergens, the conclusions highlight different issues that can be addressed at policy level:

- This report focuses on the RA concerning the allergenic potential of GMOs and proteins expressed by them. There may be other concerns related to the GMO and/or the activity, that may require additional containment measures. When questioning the adequacy of certain contain measures, it should be clear in the first place why they were selected and against which risk they are expected to provide protection. *E.g.* the measures to protect the worker possibly exposed during operation may be different from measures to contain the organisms and prevent spread of GMOs beyond containment. This may result in a higher containment level than inspired purely by the allergenic potential.

- In the case-by-case approach for GMO evaluation it is usually straightforward to determine if a hazardous substance is produced based on its characteristics. However, in the case of the allergenic potential, the hazard is predominantly determined by the predisposition of the individual. Only considering the characteristics of the substance is therefore insufficient. In the absence of the ability to establish thresholds and thereby safety margins, another reference system can be useful for applicants and assessors. Based on methodology used for the allergenic potential of chemicals, a classification method is proposed.

- Recombinant allergens may, in contrast to their natural counterparts, be prepared with very high purity and in high concentrations. Caution with these products is therefore warranted. The GMO regulations cover steps in the process in which exposure to allergens is usually limited through the containment of the producing organism. Extractions from natural allergen sources or purification steps after a recombinant allergen has been produced, may pose a much higher risk. Such activities not involving viable GMOs are solely governed by workers protection legislation and not by GMO regulations.

- In addition to the allergenic potential of the protein, also expression and exposure need to be taken into account when determining an adequate containment level. As such it is not required to restrict working with GMOs producing allergens to a BSL II level. Some activities (*e.g.* without expression or excretion of the allergen) could occur at BSL I level. In addition even in BSL II some specific requirements may need to be added (*e.g.* gloves). By detailing the activity to more precise steps, adequate and cost-efficient containment measures can be better identified.

- Making employees aware of the potential risk is important. Most adults know to which sources they are allergic, as sensitisation often occurs in childhood. Occupational sensitisation, however, usually occurs in adulthood. Extra precautions or refraining from 'risky' activities may substantially reduce the possibility of an adverse allergic reaction. In addition medical surveillance and presence of an emergency kit may be useful to quickly respond to a developing disease or emergency situation, should exposure become reality.

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Annex: Current state of knowledge

1. The immune system

The immune system is the mechanism that defends the body from invading organisms and molecules. It is a layered defence system:

1) The first barrier against infection is the **physical barrier** formed by the cell layers of the skin and the mucosa of the respiratory and gastrointestinal tract. The tightly joined epithelial cells prevent microorganisms from penetrating underlying cells. Also, mechanical mechanisms are used to clear the pathogen by coughing and sneezing, and via tears and urine. Organisms entering the nose often cause the nasal surfaces to secrete more protective mucus. Furthermore, the skin and mucosa excrete enzymes, also found in tears that increase the protection potential. Additionally, the acidity in the stomach prevents many pathogens to invade the body. The bacterial flora in the genitourinary and gastrointestinal tracts reduces the growth of pathogens by outcompeting for food and inducing changes in the environment.

2) The second defence layer is the **innate immune system** or non-specific immune system that provides an immediate, but non-specific response. The trigger is either the recognition of pathogens by pattern recognition receptors, or alarm signals produced by injured cells.

3) The third layer of protection, the **adaptive immune system**, is activated by the innate response, and is a pathogen and antigen specific response.

Both the innate and adaptive immune systems have cell-mediated and humoral components. Whereas in the adaptive immune system exposure leads to immunological memory allowing for a faster and stronger reaction upon future exposures, the innate immune system has no such mechanism. However, recent findings show that the distinction between the two types of the immune system is not that clear (Vivier *et al.*, 2011).

The immune response is regulated by cytokines that are released and responded to by cells of the immune system. Cytokines include interleukins, interferons, and growth factors.

1.1 Innate immune system

The humoral immunity component of the innate system is mediated by macromolecules found in extracellular fluids, such as blood and lymph. Injured or infected cells release the signalling molecules eicosanoids and cytokines as a first response to infection. These signalling molecules recruit immune cells to the site of infection. After removal of the pathogen they promote healing of any damaged tissue.

The complement system is the major humoral component of the innate immune response. It consists of about 25 proteins that act in a biochemical cascade that attacks the surfaces of foreign cells. The response is activated by complement binding to antibodies that have attached to the invading organisms or the binding of complement proteins to carbohydrates on their surfaces, followed by rapid killing.

The cellular immunity component consists of leukocytes (white blood cells). They act like independent, single-celled organisms and are the second component of the innate immune system. The innate leukocytes include the phagocytes (macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils, and natural killer cells. The receptors of these cells are pattern recognition receptors that recognise broad molecular patterns found on pathogens. In consequence, this response is non-specific. Mast cells reside in all connective tissues. Their activation and degranulation releasing

reactive substances that kill microorganisms, contribute to a large extent to inflammation at the site of infection.

1.2 Adaptive immune system

While the innate response acts immediately (within hours) and slows down the spread of the infection, it triggers the adaptive immune response that may last for days to weeks. This response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called antigen presentation. The receptor cells, the lymphocytes (B cells and T cells), have on their surface receptors that are highly pathogen-specific.

B cells are involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response. In B cells the cell-surface receptors for pathogens are immunoglobulins (Ig), whereas for T cells one speaks of T cell receptors (TCR).

The first time that an adaptive immune response is performed to a certain organism or substance, is referred to as the primary immune response. Any following reaction to the same organism is called a secondary immune response.

T cells recognise antigens only after they have been processed and presented in combination with a receptor called a major histocompatibility complex (MHC) molecule. Killer T cells only recognise antigens coupled to Class I MHC molecules, while helper T (T_H) cells only recognise antigens coupled to Class II MHC molecules. Killer T cells kill cells that are infected with pathogens, or are otherwise damaged or not functioning, by releasing cytotoxins. Helper T cells regulate both the innate and adaptive immune responses. After activation, they release cytokines enhancing the microbiocidal function of macrophages and the activity of killer T cells and B cells.

1.2.1 B Cell Response

After exposure to an antigen, B cells differentiate into plasma cells whose primary function is the production of antibodies (immunoglobulins), which are presented on the surface of the B cell. Recognition happens without processing of the antigen (Fig. 1). This antigen/antibody complex is taken up by the B cell, processed by proteolysis into antigenic peptides and displayed on its surface bound to MHC class II molecules. This complex attracts the matching T helper cell that releases lymphokines (cytokines) and activates the B cell to divide. The B cell offspring (plasma cells) secrete millions of copies of the antibody that recognises this antigen. These antibodies circulate in blood plasma and lymph, bind to pathogens expressing the antigen and mark them for destruction by complement activation or for uptake and destruction by phagocytes.

The cytokine interleukin 4 (IL-4) will stimulate the B cell to produce IgE.

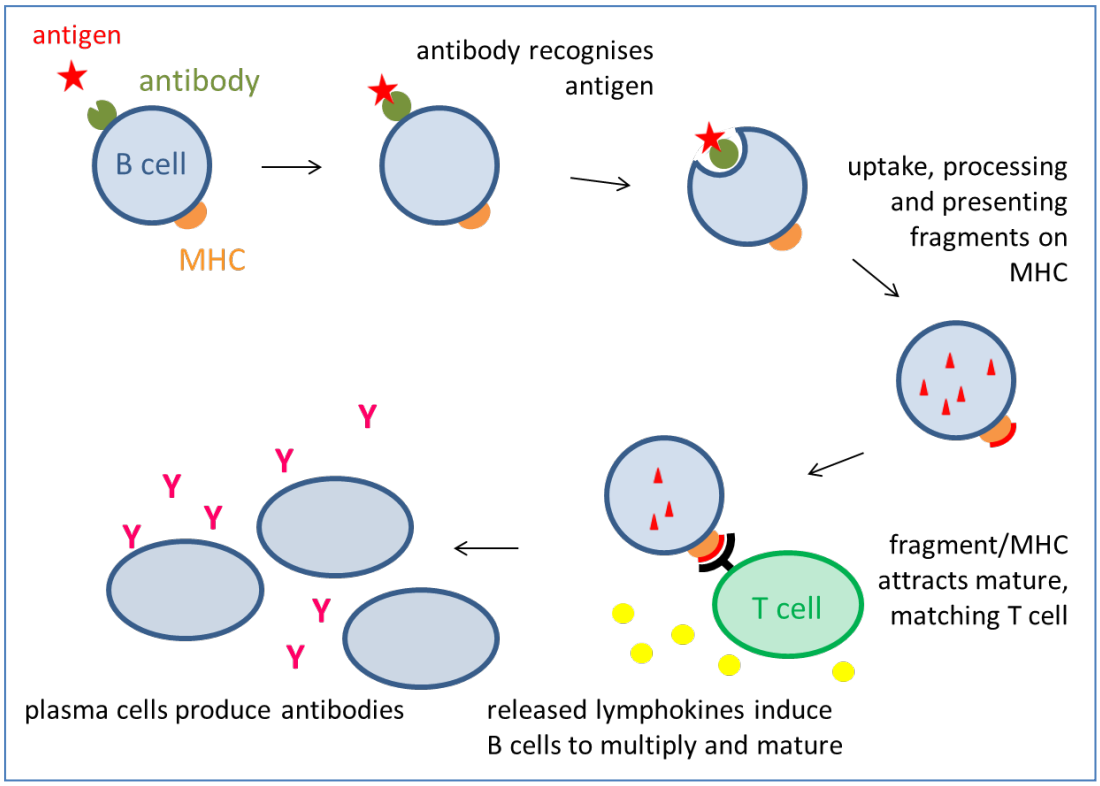

Fig. 1 B cell response
After NIAID[38], by Jeanne Kelly

1.2.2 T Cell Response

After contact with the antigen, naïve helper T cells (T_H0) differentiate into inflammatory T_H1 cells, helper T_H2 cells or pathogenic T_H17 cells, depending upon the cytokines in the environment, which is influenced by the antigen and also by the preceding innate response. Cytokines produced by T_H1 cells activate macrophages and participate in the generation of cytotoxic lymphocytes or natural killer cells, resulting in a cell-mediated immune response (Fig. 2). In contrast, cytokines produced by T_H2 cells help to activate B cells, resulting in antibody production. The helper T cell types may inhibit each other (Fig. 3). The regulating mechanism directs the immune response in function of the type of threat: cell-mediated responses for intracellular pathogens or antibody responses for extracellular pathogens.

Depending on the type of cytokines that T_H2 cells produce the B cells will switch to different classes of antibody production adapting to the different types of pathogens/substances. *E.g.* the cytokine interleukin-4 (IL-4) will stimulate the B cell to produce IgE that is involved in allergy.

When B cells and T cells are activated and begin to replicate, some of their offspring become long-lived memory cells. Memory cells will induce the secondary immune response upon the next contact with the invader.

[38] The National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA

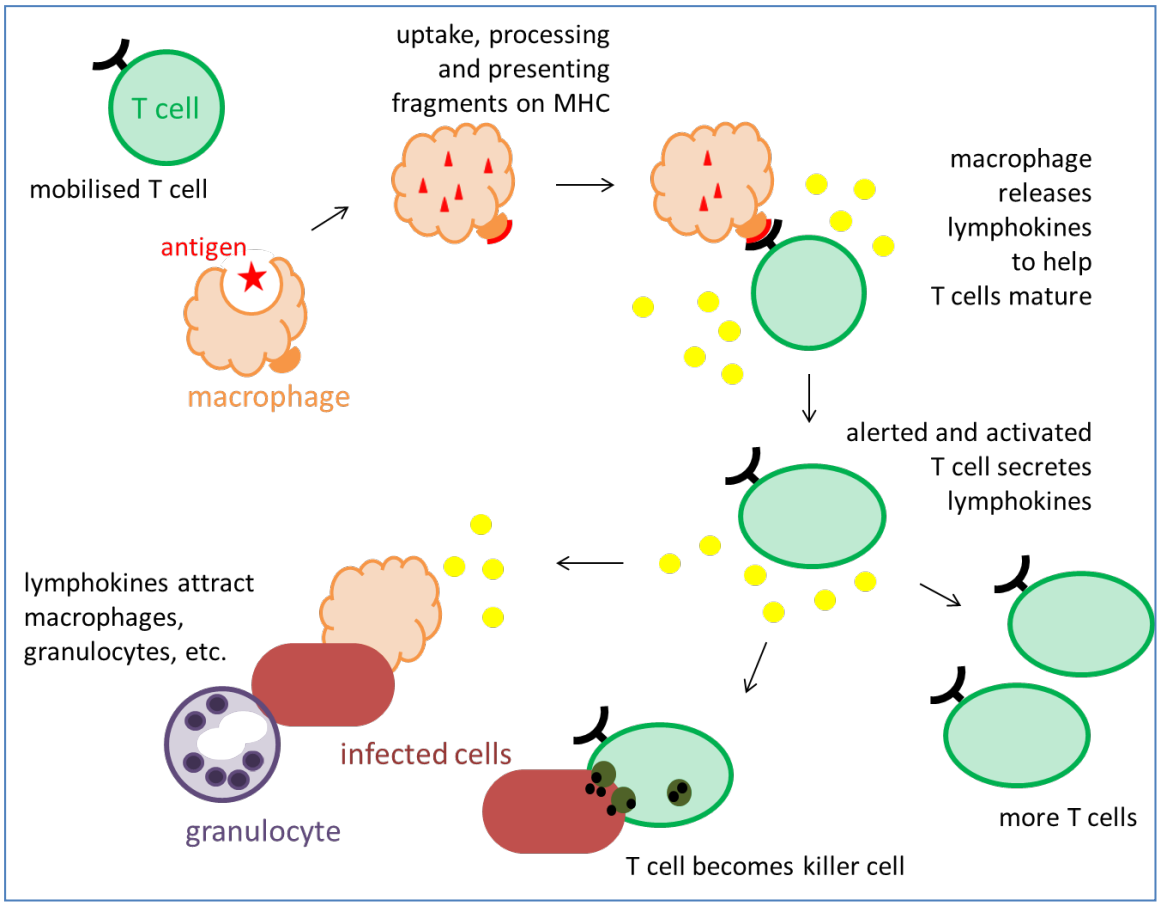

Fig. 2 T cell response
After NIAID, by Jeanne Kelly

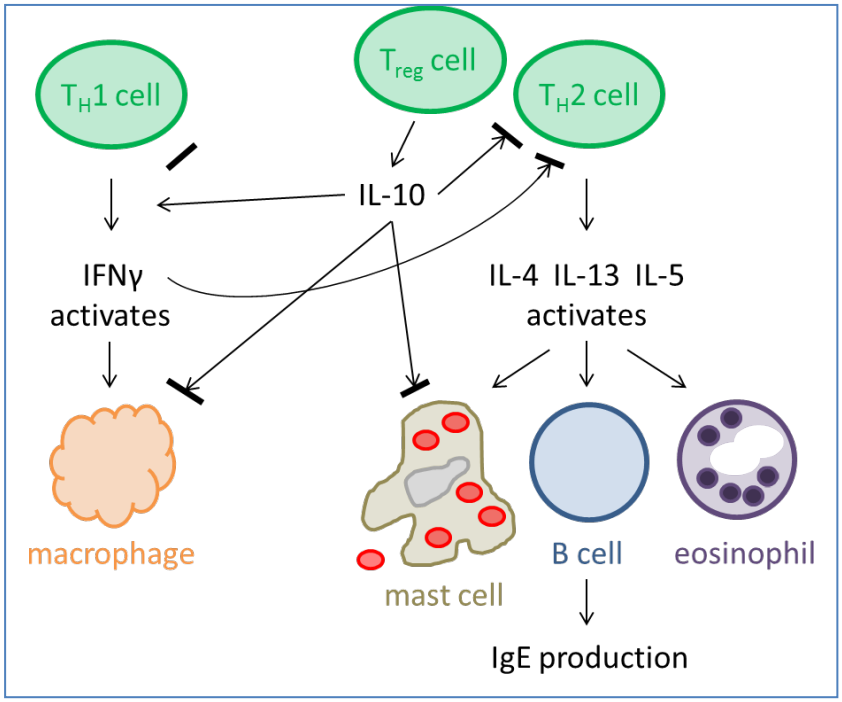

Fig. 3 Selection and regulation of the most effective immune response
Adapted from Mayer & Nyland (2010)

1.2.3 Interaction with other cells of the immune system

Dendritic cells and macrophages are antigen-presenting cells (APCs) that express class II MHC molecules. In the primary immune response dendritic cells and macrophages are the first to present antigens to helper T cells.

- **Dendritic cells**, that are found in skin tissue and other tissues in direct contact with the environment (inner lining of the respiratory and gastrointestinal tract), are the most effective APCs. Upon infection they engulf microorganisms and leave the tissue. During migration they 'digest' the organism into antigens, bind them on the MHC molecules and by displaying this complex they act as messengers to call for an adaptive immune response activating T helper cells. Dendritic cells can activate both memory and naive T cells.

- **Macrophages** are derived from monocytes that travel in the blood to tissues where they mature. They are equipped for phagocytosis. They are not as effective in presenting antigens to naïve T cells but they very effectively activate memory T cells. Likewise B cells are very active in presenting antigens to memory T cells.

- **Natural killer cells** (NK cells) are lymphocytes of the innate immune system, but play a role in adaptive immune response as well. NK cells respond to cytokines released by virally infected cells and to tumour formation. They contain granules with proteins such as perforin and proteases to kill the target cells. This functions in the absence of antibodies, but they also recognise antibody-coated cells. NK cells also produce an array of cytokines that gives them a regulatory function. Activation of NK cells, which is regulated by activating and inhibitory receptors, has an effect on macrophages, dendritic cells and neutrophils. This is followed by an antigen specific T and B cell responses. Also NK cells have the capacity to memorise (Vivier *et al.*, 2011).

- **Natural killer T cells** (NK T cells) are a subset of T cells that recognise lipids and glycolipids presented by CD1d antigen presenting molecules instead of peptide-MHC complexes.

- **Neutrophils**, the most abundant type of granulated white blood cells, are a type of phagocyte, capable of ingesting microorganisms or particles, and are normally found in the blood stream. By releasing their granules, antimicrobial proteins are freed that help fight infection. After digestion of pathogens, predominantly bacteria, they are often cleared themselves by macrophages.

- **Mast cells** and **basophils** are granulated cells that contain histamine and heparin, an anticoagulant and other substances. Mast cells can be activated by direct injury, binding of IgE receptors (in allergic reactions), or by activated complement proteins. They then degranulate and release histamine and other hormonal mediators, starting the inflammatory process. Mast cells are also involved in wound healing. Basophils circulate in the blood and have a role in both parasitic infections and allergies. Like mast cells they have receptors on their cell surface that bind IgE.

- **Eosinophils** are a fourth type of granulocytes. They circulate in blood and migrate to inflammatory sites in tissues, or to sites of parasitic worm infections in response to chemokines and leukotrienes, and are activated by cytokines released from T_H2 cells.

1.2.4 Immunoglobulins

An immunoglobulin or antibody is a large Y-shaped glycoprotein, encoded by genes that are cut, spliced and modified to produce numerous variants of the basic receptor. Each variant recognises one particular part (epitope) of a particular antigen present on pathogens or any foreign substance, allowing binding together with precision. This high affinity binding of the immunoglobulin with the antigen makes the mechanism very effective even when the antigen concentration is low.

Immunoglobulins can be membrane-bound attached to the surface of a B cell or can occur in a soluble form that is secreted from a plasma cell. In mammals there are 5 different classes of immunoglobulins (Woof & Burton, 2004):

- **IgA** is found in mucosal areas, *i.e.* the secretions by the gut, respiratory tract and urogenital tract, and prevents colonisation by pathogens. It is also found in saliva, tears, and breast milk.
- **IgD** is an antigen receptor attached to B cells that have not been exposed to antigens. It activates basophils and mast cells to produce antimicrobial factors.
- **IgE** protects against parasitic infections and is involved in allergy by binding to allergens. It triggers histamine release from mast cells and basophils.
- **IgG** provides the majority of antibody-based immunity against invading pathogens. It is the only antibody capable of crossing the placenta to give passive immunity to the foetus.
- **IgM** is either present on the surface of B cells as a monomer or in a soluble form as pentamers with many high affinity binding sites (high avidity). It is to eliminate pathogens in the early stages of B cell mediated (humoral) immunity.

The Fab (fragment, antigen binding) regions on each of the Y arm can bind to the antigen. The Fc (fragment, crystallisable) region at the base of the Y modulates immune cell activity: the Fc region ensures that each antibody generates an appropriate immune response for a given antigen, by binding to a specific class of Fc receptors. There are 2 receptors for IgE present on B cells: the low-affinity IgE receptor (FcεRII) and the high-affinity receptor FcεRI (Gould & Sutton, 2008; Woof & Burton, 2004). This FcεRI receptor is of such high affinity that binding of IgE molecules is essentially irreversible.

1.2.5 Tolerance mechanisms

Humans are continuously exposed to harmless antigens (food, commensal micro-organisms and environmental antigens). Also, the human body 'recognises' its own antigens. Furthermore, an immune response against pathogens must be controlled to prevent excessive damage to body tissues (inflammation). Regulatory T cells (Treg) are found to be key in regulatory mechanisms for tolerance to these antigens, including allergens (Agua-Doce & Graca, 2012; Josefowicz *et al.*, 2012). These cellular mechanisms persistently patrol the body preventing the onset of inflammation. While knowledge is increasing, the molecular mechanisms in Treg cell–mediated immune regulation is still limited. Many genes and suppression molecules are involved. And it is not clear yet how the immunosuppressing activity of Treg cells is modulated to allow for an effective response to *e.g.* an infectious microorganism and is resumed after clearance.

Two cytokines are very important in regulatory T cell function: transforming growth factor (TGF-β) and IL-10. Some natural Treg cells (nTreg; originating in the thymus) produce the transcription factor forkhead box protein 3 (Foxp3) and TGF-β. TGF-β induces T cell activation to become Treg cells that express Foxp3 (peripherally induced Treg cells; iTreg). Foxp3, through the regulation of expression of many genes, specifies and maintains the type of Treg cells. It prevents differentiation of Treg precursor cells into effector T cell lineages. Foxp3 represses production of pro-inflammatory cytokines by Treg cells. TGF-β also impairs differentiation to T_H2. T_H2 cells, that normally produce IL-4, are consequently outnumbered, and therefore the B cell class switch to IgE is prevented in favour of IgA and IgG production, avoiding inflammation.

Also the anti-inflammatory cytokine interleukin-10 (IL-10) plays an important role in tolerance induction (Agua-Doce & Graca, 2012; Couper *et al.*, 2008). IL-10 is produced by a variety of cells including Treg cells and to a lesser extent T_H2 cells. Through its suppressive effects on dendritic cells and macrophages, it inhibits various inflammatory pathologies. It also regulates *e.g.* T_H2 responses to prevent the overproduction of IL-4, IL-5 and IL-13, and T_H1 cells to produce IL-12 and interferon-gamma (IFN-γ). IL-10 production by one cell population can therefore affect the ability of other cells to make IL-10, in this way allowing for IL-10-producing cells to regulate each other. Different types of effector cells, including T_H2, may produce IL-10 at the end of the immune response in a mechanism to limit their inflammatory behaviour. Both IL-10-secreting Treg cells, and nTreg cells, that express Foxp3, have a comparable ability to inhibit the proliferation of T_0 cells.

Besides conventional T cells, also NK T cells are important players in defining the outcome of immune responses (Agua-Doce & Graca, 2012). NK cells likewise express a series of activating and inhibitory receptors that is calibrated to ensure tolerance to body antigens and at the same time provides for a suitable response to viral infection and tumour development (Vivier *et al.*, 2011).

1.2.6 Development of the immune system

During pregnancy the T cell system of the foetus needs to mature efficiently to be ready to cope with a diversity of substances and organisms at birth. On the other hand, the potential for maternal-foetal allogeneic reactions *in utero* must be minimised. The human foetus has a T cell system that is reduced in T_H1 functional capacity and more T_H2 cytokines are present. T_H1 function is important for the defence against virus and other pathogens, but also plays a role in tissue or organ rejection. The latter would endanger the compatibility between mother and foetus. The mother exerts the same shift towards T_H2 not to reject the foetus. At birth and in the days after birth the balance rapidly shifts towards T_H1 cytokines.

2. Allergy

Allergy is a disorder that is, strictly speaking, different from hypersensitivity in that it requires specific recognition by the immune system. The body reacts to normally harmless substances in the environment. This overreaction of the adaptive immune system can be antibody-mediated or cell-mediated.

2.1 Allergic reactions

For practical reasons typically four types of allergic reactions are discriminated (Table 1).

For this report mainly type I and to a lesser extent type IV reactions are relevant as in CU conditions the allergenic substances are recombinant proteins, to which workers are exposed to predominately via inhalation. In skin contact allergy usually low molecular weight substances are responsible rather than proteins.

Table 1 Types of allergic reactions

Type I hypersensitivity	An immediate or anaphylactic reaction on re-exposure to a specific antigen. Exposure may be by ingestion, inhalation, injection, or direct contact. It is mediated by IgE, which triggers degranulation of mast cells and basophils when cross-linked by the antigen. An immediate (within seconds or minutes) and late-phase reaction (within 6-8h) are discerned, the latter being characterised by infiltration of eosinophils and other inflammatory cells. Examples are allergic asthma, anaphylaxis, allergic conjunctivitis and allergic rhinitis (hay fever).
Type II hypersensitivity (cytotoxic hypersensitivity)	Antibodies bind to antigens on the patient's own cells, marking them for destruction. Damage is caused by neutrophils and NK cells. The hemolytic reaction to penicillin is a typical example: the drug can bind to red blood cells, leading eventually to the elimination of the cells.
Type III hypersensitivity	These reactions happen when immune complexes (aggregations of antigens, complement proteins, and IgG and IgM antibodies) are formed and not properly cleared and are deposited in various tissues. Neutrophils and the complement system are activated. This induces an inflammatory response. Examples are the initial phase of farmer's lung and serum sickness.
Type IV hypersensitivity (delayed type hypersensitivity, delayed allergy)	This is a cell-mediated response. It takes between two and three days to develop. The reactions are mediated by helper T cells, monocytes, and macrophages. Contact dermatitis is an example of a Type IV allergy, as are many types of drug allergy.

2.2 Mechanisms of allergy development

An antigen triggers the production of one or more antibodies. An allergen may be defined as a substance capable of causing an allergic reaction. They are generally divided into two groups:

- High molecular weight allergens (>5000 dalton) are usually proteins that induce an IgE-mediated, type I allergic reaction;
- Low molecular weight allergens (<1000 dalton) are most often chemicals reacting with T cells.

Atopy is a predisposition towards developing certain allergic reactions (type I) and is partly genetically determined next to environmental factors. Not everyone reacts to an antigen exposure with an allergic response. Some people suffer from disease where most others do not show any symptom. Allergy is believed to result from a disturbed balance between the different T cells. T_H1, T_H2, T_H17 and Treg cells regulate each other through positive and negative feedback mechanisms.

T_H1 cells produce cytokines like IFN-γ, that, amongst others, activate macrophages and stimulate B cells to produce complement-binding antibodies and antibodies that enhance binding on micro-organisms for phagocytosis. The macrophages in their turn stimulate T_H1 cell differentiation and inhibit the proliferation of T_H2 cells.

T_H2 lymphocytes produce cytokines such as IL-4, IL-5, and IL-13. IL-4 and IL-13 stimulate B lymphocytes to produce IgE that triggers mast cells to release mediators of inflammation. IL-13 also stimulates increased mucus production. IL-5 is important for eosinophil infiltration and activation. IL-4 promotes further T_H2 cell differentiation and inhibits the proliferation of T_H1 lymphocytes.

When T_H2 cells predominate over T_H1 cells, this results in a Type I hypersensitivity reaction. The mechanism behind the shift from T_H1 to T_H2 is not known. However, dominant regulatory mechanisms with Treg cells in a central role may be essential (Agua-Doce & Graca, 2012; Hawrylowicz & O'Garra, 2005; Josefowicz *et al.*, 2012).

The origin of the imbalance may go back to a delayed or impaired T_H1 maturation soon after birth. This is expected to leave the individual with a potentially life-long immune imbalance (Dietert & Piepenbrink, 2008). The retention of the foetal immune imbalance may be the reason for early asthma development. Food and environmental factors exposed to both mother and the neonate influence this T_H1 maturation process next to genetic factors.

2.2.1 Sensitisation

Sensitisation is the induction of a specialised immunological memory in an individual by exposure to an allergen. The immune system responds with a specific cellular and/or humoral response (IgE, IgG, etc.) to the allergen in question, a process that by itself does not cause any symptoms.

In this and the next paragraphs the processes leading to type I allergic reactions are described.

When an allergen is encountered for the first time (sensitisation phase), it is taken up by an APC, *e.g.* a dendritic cell or B cell (Fig. 4) (Galli *et al.*, 2008; Hawrylowicz & O'Garra, 2005; Prussin & Metcalfe, 2006). These cells process the antigen and present the peptide-MHC complex to the T_H2 cell. This is then recognised by the TCR on this cell. As a result the T_H2 cell produces cytokines (IL-4, IL-13) and the CD40 ligand. Both are needed to stimulate B cells. The cytokines activate transcription at a specific immunoglobulin locus. Ligation of CD40 on B cells activates DNA switch recombination (IgE class switching). The B cells start the production IgEs and excrete them in the blood. The IgE then binds to a high-affinity receptor FcεRI on the surface of mast cells and basophils (FcεRI is expressed on mast cells and basophils, as well as on APCs, monocytes, eosinophils, platelets and smooth-muscle cells).

2.2.2 Elicitation

Only after the second and subsequent exposure an allergic reaction is induced (elicitation, allergy phase). At the next encounter with the same antigens, these will bind to the corresponding IgE coated on the surface of the sensitised mast cells or basophils (Fig. 5). Cross-linking of the IgE and FcεRI receptors occurs when more than one IgE-receptor complex interacts with the same allergenic molecule, and activates the sensitised cell. The aggregation of FcεRI triggers a complex intracellular signalling process resulting in the secretion of biologically active molecules. In their granules the sensitised cells keep preformed molecules (causing early-phase reactions), but they also form new substances (causing late-phase reactions). They release histamine and other inflammatory chemical mediators (cytokines, interleukins, leukotrienes, and prostaglandins) from their granules into the surrounding tissue causing several systemic effects, such as vasodilation (widening of blood vessels), mucous secretion, nerve stimulation and smooth muscle contraction (*e.g.* in the longs obstructing airflow resulting in wheezing). This leads to erythema (reddening) of the skin, rhinorrhea (runny nose), dyspnea (shortness of breath), itchiness, urticarial (hives), vomiting, diarrhoea, and anaphylaxis. Six to 24 hours later this is followed by a persistent oedema (swelling from fluid accumulation) and a leukocytic influx (late-phase reaction). In the lungs it is believed to play a major role in the genesis of persistent asthma.

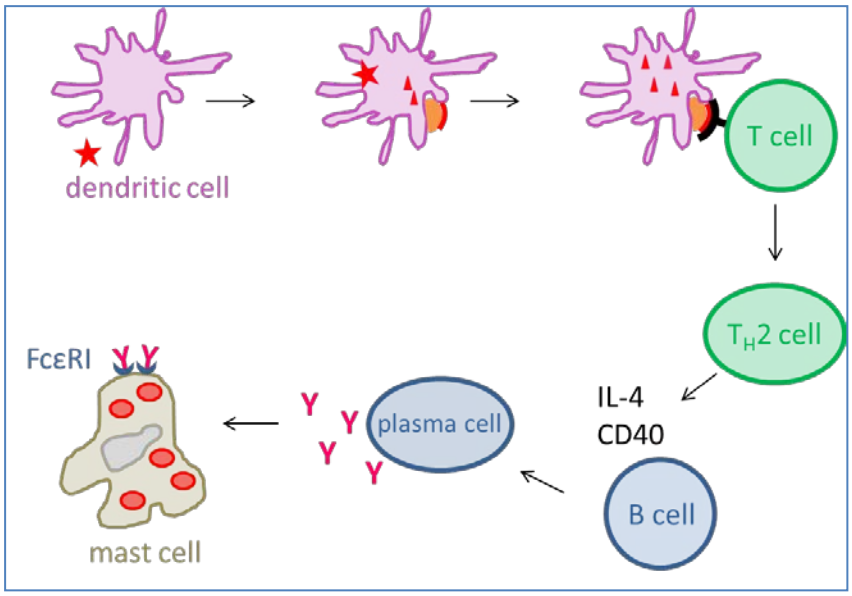

Fig. 4 Allergic reaction: Sensitisation
After Hawrylowicz & O'Garra, 2005

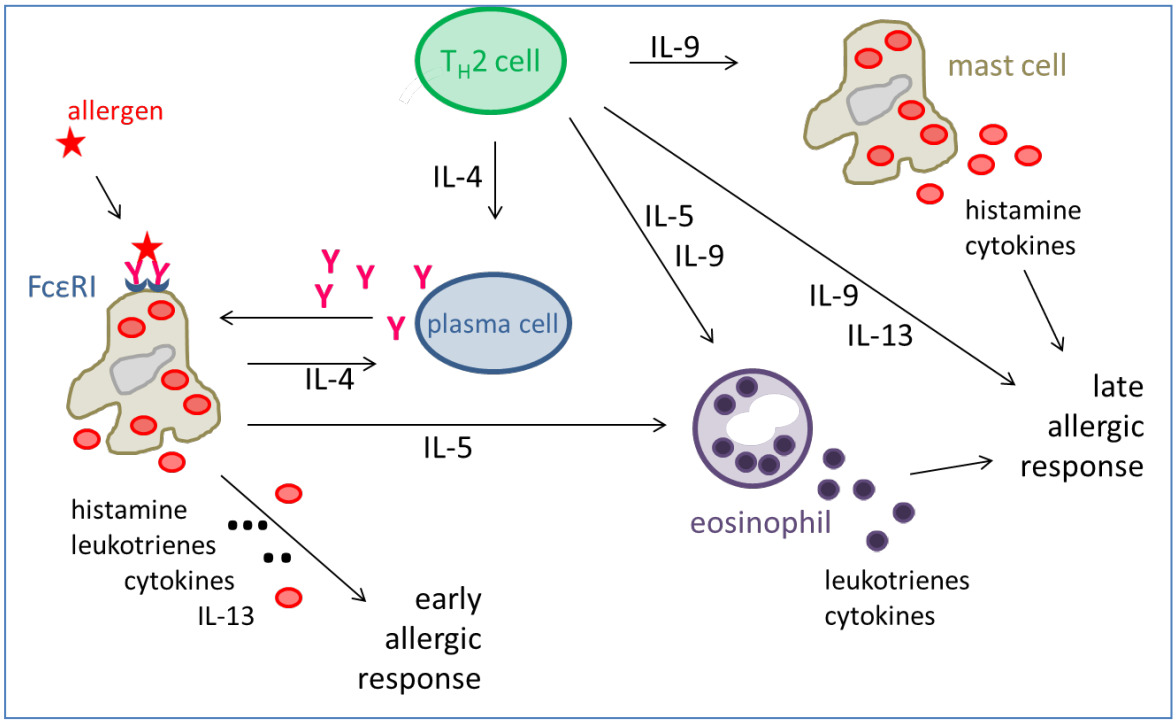

Fig. 5 Allergic response: Elicitation
After Hawrylowicz & O'Garra, 2005

When allergen exposure is continuous or repetitive, inflammation persists. This chronic allergic inflammation is linked with changes in the structural cells at the affected sites (tissue remodelling). Examples are increased vascularity, the development of nasal polyps, and changes in the barrier function of the affected epithelia.

Depending on the individual, the allergen, and the mode of introduction, the symptoms can be system-wide (classical anaphylaxis), or localised to particular body systems: *e.g.* asthma to the respiratory system, eczema to the dermis. Also, total IgE levels are influenced by age, genetic makeup and race,

immune status, environmental factors (*e.g.* season of the year for hay fever), and disease process (Prussin & Metcalfe, 2006).

2.2.3 Further considerations

The likelihood of developing clinically significant sensitisation and the mounting of a T_H2 response is affected by a person's genotype, type of allergen, allergen concentration in the environment and whether exposure occurs together with agents that can enhance the sensitisation process (Galli *et al.,* 2008; Mohapatra & Lockey, 2001).

The allergic reaction resembles the body's reaction on parasitic infections (helminths) or bites of *e.g.* ticks (Galli *et al.,* 2008). When chronically infected by parasites, the body turns on immunological mechanisms that down-regulate the inflammation and tissue damage. Treg cells are involved secreting IL-10 that has immunosuppressive and anti-inflammatory effects.

Allergens may mislead the immune system inducing the same reaction. However, the immunosuppressive response seems to fail. This has led to the 'hygiene hypothesis' that says that reduced exposure to parasitic infections and to other pathogenic and non-pathogenic microorganisms in the developed world impedes the normal development of an immune response. The result is a bias towards T_H1 cells instead of T_H2 cells and Treg cells controlling potentially harmful immune responses. Predisposed persons therefore run the risk to respond to otherwise harmless antigens by developing a T_H2 cell-type response.

TGF-β and IL-10 both protect from allergic diseases, as discussed previously. In contrast, allergic individuals express lower levels of IL-10 that normally inhibits mast cell degranulation.

Although initially the focus of understanding was on an imbalance between T_H1 and T_H2 response, it is now clear that the causes for type I allergies should be searched more upstream, concentrating on genes involved in the control of inflammation and innate immune responses. Both nTreg cells and antigen-driven IL-10-secreting Treg cells have been implicated in the regulation of allergen-induced T_H2 responses in humans. In patients with allergies the function of nTreg cells may be reduced or altered compared with normal healthy individuals (Hawrylowicz & O'Garra, 2005). Also, a rare mutation in the gene encoding Foxp3, which is expressed at high levels by nTreg cells, results in allergic dysregulation. Finally, nTreg cells inhibit T_H2-cytokine responses less effectively in allergic patients. In atopic patients there is an imbalance between IL-10-secreting Treg cells and IL-4-secreting, allergen-specificT_H2 cells, in favour of IL-4.

Due to reduced functioning of the epithelial barrier, a person who has at first only one allergic disorder, may develop others later on (Galli *et al.,* 2008). The body is in this way challenged by increased exposure to the original allergens and additional allergens. APCs that capture the allergen with specific IgEs and process it, may promote the development of T_H2 cell responses to other epitopes of the allergen for which sensitisation already exists or to other allergens that are being processed in parallel by the same APCs.

In allergic persons the increased IgE level may amplify the reaction of mast cells and basophils (Galli *et al.,* 2008). Also, some IgE may aggregate antigen-independently after binding to FcεRI. In this way an immune reaction is evoked without exposure to the antigen, which may explain why symptoms persist even in the absence of the specific antigen. Moreover, independently from IgE, mast cells may contribute to allergic inflammation (Galli *et al.,* 2008). Basophils produce IL-4 through both IgE-dependent and IgE-independent mechanisms (Prussin & Metcalfe, 2006). Basophils are a dominant and rapid source of IL-4 in both allergen- and helminth-specific responses. Eosinophils play a pro-

inflammatory role. Mediator molecules released by eosinophils are believed to cause mucosal inflammation, airway remodelling and subsequent bronchial hyper-responsiveness.

The genes and mechanisms that are responsible for allergic disease are only just being discovered (WAO, 2011). They may be involved in the innate immune system, have an epithelial barrier function, regulate the immune response, and determine the tissue response to chronic inflammation. Also, epigenetic mechanisms may contribute to gene-environmental interactions and trans-generational effects (Koppelman & Nawijn, 2011; Pacheco, 2012; WAO, 2011).

Hygiene hypothesis

The hygiene hypothesis was postulated by David Strachan based on observations of increasing prevalence of hay fever, asthma and childhood eczema in recent decades (Strachan, 1989). The author noticed an inverse relation between family size and position in the household in childhood and the occurrence of hay fever.

This hypothesis says that improved hygiene especially early in life, as is often found in developed vs. developing countries and urban vs. rural areas, can lead to a decline in infectious stimuli that are necessary for the proper development of the immune system. Large families and farm upbringing would protect children from developing allergies especially asthma.

The decrease in exposure to infectious stimuli due to improved hygiene, vaccination, and use of antibiotics (that reduce intestinal biodiversity), may result in an imbalance between T_H1 (associated with bacterial and viral infections and autoimmune diseases) and T_H2 (associated with helminth infections and allergic diseases) immune responses. However, not all pathogen or parasitic infections have this protecting capacity (Brooks *et al.*, 2013).

A strong T_H2 response is not the only factor causing disease as helminth infection does usually not coincide with allergy (Yazdanbakhsh *et al.*, 2002). Several studies found that heavily and chronically parasitised patients are somehow protected from mast cell degranulation and inflammatory responses, whereas light helminth infections lead to allergy. Altered immunoregulatory mechanisms may be important. Microorganisms may modulate the Treg cell functioning or other regulatory mechanisms.

Although some contradicting findings have been reported, the hygiene hypothesis may still be valid in general. However, other factors may be involved as well (Brooks *et al.*, 2013). It may be useful to identify the distinct molecular patterns on parasite and pathogen that have immunomodulatory effects in the wealth of organisms and substances people are exposed to (Yazdanbakhsh *et al.*, 2002).

2.3 Influencing factors

2.3.1 Atopy

Atopy may be defined as a genetically mediated predisposition to an excessive IgE reaction. It is a personal and/or familial tendency, usually in childhood or adolescence, to become sensitised and produce IgE antibodies in response to ordinary exposure of allergens, usually proteins (WAO/EAACI). The syndrome is characterised by a tendency to be “hyperallergic”. Atopic patients usually may suffer

from more than one kind of type I hypersensitivity reaction, such as eczema (atopic dermatitis), allergic rhinitis (hay fever), allergic conjunctivitis, or allergic asthma.
Atopy is thought to be influenced as well by antenatal and/or postnatal exposures (Chung *et al.*, 2007).

However, contact with the allergen must occur before the hypersensitivity reaction can develop. Atopic individuals have an intrinsic tendency to develop type I hypersensitivity allergic reactions against one or several common environmental allergens (EFSA, 2010), for example pollen, dander, dust mites, certain foods, or chemical/physical irritants.

2.3.2 Haptenisation

A hapten is defined as any small molecule (<1000 dalton) that can elicit an immune response only when attached to a large carrier such as a protein. The carrier is usually an endogenous or exogenous protein to which the molecule is covalently bound (Chipinda *et al.*, 2011). Haptens may interact with body proteins or food proteins and cause these proteins to become allergenic (FAO/WHO, 2001).
Haptenisation is the reaction of an antigenic compound (a hapten) with a carrier protein in order to stimulate an immune response.

A well-known example of a hapten is urushiol, the toxin found in poison ivy sap (*Toxicodendron radicans*). Exposure is usually via the skin. When absorbed through the skin, urushiol oxidises in the skin cells to generate the actual hapten, quinone, which then reacts with skin membrane proteins to form hapten adducts. After sensitisation and a subsequent second exposure, typical blisters are produced called urushiol-induced contact dermatitis. The response is T cell-mediated.

2.3.3 Adjuvants and environmental factors

Adjuvants are defined as non-immunogenic substances that may stimulate the immune system, without being an antigen itself, *i.e.* substances that, when co-administered with a protein increases its immunogenicity and therefore might increase as well its allergenicity (EFSA, 2010; WAO, 2011). These may act as modifiers of the microenvironment in which APCs are active, leading to increased efficiency of antigen presentation to T cells, or disturbing the balance between the various T cells. Different mechanisms may be underlying adjuvant activity; the precise nature is not fully understood.

Various substances may act as an adjuvant: lipopolysaccharides, proteins, cytokines, CpG oligonucleotides, bacterial toxins, chemicals like aluminium etc. Also so-called ‘environmental adjuvants’ like tobacco smoke and diesel exhaust particles are adjuvants for allergy development (EFSA, 2010). In a literature review Nielsen and colleagues (2007) found little evidence that indoor dust, cleaning agents, anionic and non-ionic surfactants, quaternary ammonium compounds and phthalates possess important adjuvant effects. Pollen grains, in addition to their protein allergens carry with them substances that function as adjuvants (pollen-associated lipid mediators, such as phytoprostanes (Gilles *et al.*, 2000).

Substances may as well facilitate allergic reaction by irritation: chemicals may damage airway epithelia cells resulting in an increased actual exposure to the allergen.

Indoor and outdoor pollution in general has a positive association with the prevalence of asthma and allergic diseases (Carlsten & Melén, 2012; WAO, 2011). Air pollution may induce sensitisation to common allergens. The effect may be modified by co-exposure of allergens as well as genetic constitution.

Pathogens causing disease like virus infections may enhance an allergic response. Likewise, some bacteria may interfere through endotoxins in their cell wall.

In general dietary, lifestyle and occupational factors have each contributed to an increase in allergy in recent years, pointing to the fact that not only the gene pool is involved, as this is unlikely to have changed in this short period.

2.4 Characteristics of allergens

Whether an antigen behaves as an allergen depends on the extrinsic and intrinsic properties of the antigen (Mohapatra & Lockey, 2001). The extrinsic factors are the dosage and the route of exposure. Both influence the mode of antigen presentation. The intrinsic properties of an antigen that determine its allergenicity are its epitopes, specifically the epitopes interacting with the T cells.

Indeed, for an allergen to be recognised by the immune system it needs to have parts, *i.e.* epitopes, that can interact with B and T cells. Allergenic proteins may be of diverse functionality, but they are also structurally heterogeneous. There is no single type of structure that is common to all protein allergens.

The primary structure of a protein is the amino acid sequence. This sequence determines the local substructures (α-helix or β-sheets, secondary structure), the protein fold, the domain structure, and surface structure (tertiary structure). An epitope is that part of the surface structure that on an atomic level interacts with the antibody (Aalberse, 2000). Epitopes that interact with B cells may be localised on distant parts of the amino acid sequence, but come together in the tertiary structure. T cells only recognise short peptides presented by APCs. Therefore, epitopes recognised by B cells are essentially conformational in nature, whereas epitopes recognised by T cells are linear.

But in individuals prone to an allergic response the mechanism is directed to a T_H2-like response. T cell activation depends on how strong the peptide epitope is interacting with the T cell. This is controlled by:

- The antigen concentration and the type of APC.
- The cytokine milieu of the T cell, which is important when the latter is being triggered by the APC with a specific antigenic epitope.
- The host immune-response genes, which may bias the overall immune responsiveness of an individual in favour of a T_H2-like response (Mohapatra & Lockey, 2001).

Allergens further may have two properties: the property to sensitise, that is to induce the immune system to produce IgE specific to the allergen, and the property to elicit an allergic reaction. Some proteins, however, are known to elicit allergic symptoms but do not usually sensitise (Aalberse, 2000).

2.5 Cross-reactivity

Sometimes an antigen is able to bind with an antibody that was raised to a different antigen. This is called cross-reactivity. The definition of cross-reactivity is based on immunologic recognition: two allergens are cross-reactive if there is a single antibody (or T cell receptor) that reacts with both. (Aalberse *et al.*, 2001).

To cross-react the proteins have to show similarities in the primary structure (amino acid sequence) as well as in the tertiary structure (folding of the protein). However, this is not always necessary. In glycoproteins the determining factor may be shared glycans irrespective of the protein structures (cross-reacting carbohydrate determinant) (Aalberse, 2000; Aalberse *at el.*, 2001). The reverse,

proteins with a similar fold are not necessarily cross-reactive. This is partially due to immunologic tolerance induced by autologous proteins with a similar structure.

Cross-reactivity exists between antigens from a variety of distinct sources. The underlying cause may be shared epitopes on multivalent antigens or a conformational similarity of epitopes. In the first case the binding affinity is the same as for the original antigen, in the second case antibodies would bind with lesser affinity (Weber, 2001). The author provides extensive tables of organisms and describes the major allergens and potential cross-reactivity. Cross-reactivity reflects taxonomy in the majority of cases. The antigen profilin, however, is an example of a highly conserved protein and appears in most eukaryotic organisms. It is often responsible for pollen and food allergen cross-reactivity (Hauser *et al.*, 2010; Reindl *et al.*, 2002; Van Ree *et al.*, 1994b; Weber, 2001). This phenomenon has the complication that sensitisation to a given food allergen can occur via the respiratory route or via skin exposure (Taylor *et al.*, 2009). Other examples are latex proteins that cross-react with fruits and vegetables, because of the presence of common IgE epitopes in several classes of proteins (*e.g.* class I chitinase, lipid transfer protein) (Gawchik, 2011). Cross-reactivity between latex and pollen was demonstrated to be caused by glycoproteins where the carbohydrates contribute to the IgE-binding capacity of epitopes (Fuchs *et al.*, 1997).

3. Routes of exposure

3.1 Ingestion (Food allergy)

Food allergies are mostly IgE-mediated type I responses. The symptoms may be gastrointestinal (oral allergy syndrome), respiratory or inflammation of the skin (atopic eczema), but also life-threatening anaphylactic responses and vasodilation. The amount of food needed to trigger a reaction also varies from person to person and from food to food.

Peanut allergy is a well-known type I hypersensitivity reaction, that may result in anaphylaxis. A major peanut allergen is *e.g.* Ara h 1 belonging to the vicilin family and Ara h 2, a 2S albumin.
Celiac disease is a combination of an autoimmune disease and a T cell-mediated immunological response triggered by gluten (gliadin, a prolamin). Patients are affected by an inflammatory process in the small intestine leading to discomfort in the digestive tract, anaemia, diarrhoea, and bone pain along with other symptoms. The disease demands lifelong avoidance of gluten from wheat, rye, barley, and related cereals (FAO/WHO, 2001).

Only a limited number of protein superfamilies account for the major part of food allergens (around 65% of plant food allergens) (Mills, 2011). These families contain structurally and biologically related proteins. The four most important plant protein families are the prolamin, cupin, Bet v 1-like, and profilin families. The animal food allergens can be classified into three main families: the tropomyosins, EF-hand proteins, and caseins (EFSA, 2010; Mills, 2011). The prolamin superfamily that is the dominant family comprises the cereal seed storage proteins, 2S seed storage albumins, cereal inhibitors of trypsin and α-amylase and the non-specific lipid transfer proteins (EFSA, 2010). Examples of the cupins are the 7S and 11S seed storage globulins. Homologues of the major birch pollen allergen, Bet v 1, are the next in line.

Ingested food and the proteins therein are susceptible to the low pH in the stomach and to proteolytic enzymes secreted into the intestinal tract, particularly pepsin, leading to degradation to polypeptides, peptides, and amino acids. Protein digestion results in loss of structural integrity. In this way also conformational epitopes are lost. Also, the absorption of substances by the gastrointestinal tract is

inversely related to the size of the molecule, meaning that it is very unlikely that whole proteins are taken up.

Proteins that may induce allergenicity tend to be more resistant to proteases. Limited proteolysis might enhance mucosal transport. Therefore, the protein aspects that are important in relation to an allergic response are solubility, stability, susceptibility to proteases, size, and the compactness of the overall structure.

3.2 Contact allergy

Contact allergy is the result of the interplay between environmental exposures and individual susceptibility. As the terminology indicates, it arises from contact of the skin or mucus with an allergen. Bothe type I (contact urticaria) and type IV (contact eczema) may occur.

As a first step the allergen has to be taken up by the epidermis. The larger the molecule the smaller the probability to be taken up by cells. Therefore, the responsible allergens are most often small molecules (type IV). Small wounds and irritating agents creating wounds will facilitate the uptake of allergens through the skin. This is then followed by sensitisation and elicitation upon later contacts.

Multiple contact allergies may develop in susceptible persons (Carlsen *et al.*, 2008). Genetic factors play a role in the increased susceptibility, whereas cross-reactions only explain a small part of the occurrences.

Several types of contact allergies are known (Brancaccio & Alvarez, 2004) and briefly described hereunder.

3.2.1 Allergic contact dermatitis

This is a delayed type of induced sensitivity (type IV) resulting from skin contact with a specific allergen. This allergic reaction causes inflammation of the skin, erythema, oedema, and blister formation. It is a cell-mediated hypersensitivity reaction. Symptoms usually develop within 24-48 hours of cutaneous or mucous membrane exposure in a sensitised person, although changes as early as 4-8 hours after contact can be seen histologically.

The responsible allergens are soluble haptens. Most of the chemicals able to provoke allergic contact dermatitis are small molecules (<500 dalton). These molecules bind to carrier proteins in the epidermis. Langerhans cells, which are situated within the suprabasilar layer of the epidermis, recognise the hapten-protein conjugates as foreign substances. They are the antigen-presenting cells within the skin: they engulf the conjugates, process them and interact in association with class-II MHCs with helper T cells. The process is controlled by cytokines and chemokines.

The most known chemicals that may induce allergic contact dermatitis are metals (*e.g.* nickel, gold, chromium), preservatives (*e.g.* quaternium-15, isothiazolinones), dyes (*e.g.* p-phenylenediamine in permanent hair dye products and temporary henna tattoos), and fragrances (in perfumes, colognes, aftershaves, deodorants, and soaps). The latex type IV allergic reaction is caused by the chemicals used to process the rubber: accelerators and antioxidants left from the original manufacturing process.

3.2.2 Photoallergic contact dermatitis

This type of dermatitis is essentially an allergic contact dermatitis (type IV) that may be induced by UV and/or visible light (Kerr & Ferguson, 2010). Hapten formation between the activated antigen and a skin protein is necessary to incite a delayed hypersensitivity reaction. The most common photosensitisers are sunscreens and topical non-steroidal anti-inflammatory drugs. No proteins are known to be the inducing agent.

3.2.3 Immunological contact urticaria

The symptoms on the skin are 'wheels' *i.e.* raised areas surrounded by a red base, due to fluid leakage from superficial blood vessels. Also, fluid leakage from much deeper blood vessels in the subcutaneous or submucosal layers may occur, most often in the face, around the mouth and in the throat, and in the abdomen. It is referred to as angiooedema.

The pathogenesis reflects a type I hypersensitivity reaction, mediated by allergen-specific IgE in a previously sensitised individual (Goossens, 2011). It may be activated through IgG- and IgM-mediated pathways (type III) (Brancaccio & Alvarez, 2004). In that case the route of contact is not the skin but usually oral or intravenous contact. The allergen may originate from food and food additives, but often the cause is unknown.

3.2.4 Protein contact dermatitis

This reaction is considered to be a combination of immediate type I and delayed type IV allergic responses. It manifests as a chronic or recurrent eczematous reaction upon contact to large proteins in foods, only possible in compromised skin.
Butchers having to eviscerate and separate of various intestinal organs, come into contact with animal gut enzymes. Likewise, bakers are exposed to α-amylase from different types of flour.

3.2.5 Systemic contact dermatitis

This disease develops after oral or parenteral exposure to an allergen in a topically sensitised individual.

Other types of dermatitis may be prevalent but are entirely independent of the immune system. Irritant contact dermatitis is the result of the direct toxic effect of an agent in contact with the epidermis. Phototoxic contact dermatitis occurs after contact of an element followed by exposure to sunlight. Phytophotodermatitis, a form of phototoxic contact dermatitis, results from contact with a light-sensitizing botanical substance and UV light. It manifests as a burning erythema that may subsequently blister. Later on a post-inflammatory hyperpigmentation may show. Examples are psoralens, isolated from the plant families Umbelliferae, Rutaceae, Moraceae, and Leguminosae.

Natural rubber latex allergies

Rubber exposure may lead to three types of reactions (Gawchik, 2011):

- Irritant contact dermatitis. It is not immune mediated, and therefore no allergy. This is the most common reaction resulting from mechanical disruption of the skin, causing dry, itchy, and irritated areas.
- A delayed (type IV) hypersensitivity reaction, *i.e.* allergic contact dermatitis. Symptoms are visible within 24-48 hours after contact and the primary allergens are residual accelerators and antioxidants (thiurams, mercapto benzathiazoles and thiocarbonates) left from the original manufacturing process.
- An immediate (type I) hypersensitivity. It is the most serious, potentially life-threatening reaction, but least common syndrome. The reaction may include rhinoconjunctivitis, asthma, and systemic reaction (perioperative anaphylaxis). It is mediated by an IgE response specific for latex proteins.

More than 200 proteins have been isolated from latex sap. Latex proteins vary in their allergenic potential. Only Hev b 1 through Hev b 14 are known to be allergenic (Gawchik, 2011). Some of them cross-react with fruit and fungi. A total of 207 latex epitopes are reported (Vaughan *et al.*, 2010). The latex epitopes include both linear and nonlinear antibody epitopes, as well as T cell epitopes.
Sensitisation may occur through inhalation, mucous-membrane contact and abraded skin, apart from contact during surgery for example (visceral/peritoneal contact).

In relation to laboratory work the avoidance of latex protein containing surgery gloves may prevent adverse reactions.

3.3 Inhalation allergy

The most well-known type of inhalation allergy or airway allergy is hay fever, a seasonal allergy, induced by inhalation of pollen from certain grasses, also known as pollinosis. Once again, allergens can be categorised as either high or low molecular weight allergens. The latter act as haptens (haptens are more relevant in causing skin reactions), the former are proteins as such, but present on particles like pollen, dust, fungi, mites, etc.
Food allergens may act as aeroallergens as well. Especially in occupational settings inhalation of food particles may affect highly sensitive individuals (*e.g.* wheat), or may sensitise workers (Martel *et al.*, 2010; Ramirez & Bahna, 2009).

3.3.1 Allergic rhinitis

Rhinitis is defined as inflammation of the nasal membranes. Allergic rhinitis is the most common cause of rhinitis. Hay fever is only one example, also allergies caused by dust and animal dander are characterised by sneezing, nasal congestion, nasal itching, and rhinorrhea (runny nose) (Skoner, 2001). The eyes, ears, sinuses, and throat may also be affected. Combined with inflammation of the eye connective tissue the disease is termed rhinoconjunctivitis. Allergic rhinitis is a type I hypersensitivity response resulting in an inflammation of the mucous membranes, triggered by an IgE–mediated response to an extrinsic protein.

When the allergenic protein is inhaled into the nose of a sensitised person, it binds to the IgE on the mast cells, present in the nasal mucosa. Upon recognition they degranulate releasing mediators such

as histamine, tryptase, chymase, kinins, and heparin. The mast cells quickly synthesise other mediators, including leukotrienes and prostaglandin D2. Through various interactions these mediators in their turn ultimately lead to the symptoms of rhinorrhea. This response develops within minutes of allergen exposure (early-phase response).

Other inflammatory cells are recruited to the mucosa, such as, eosinophils, lymphocytes, and macrophages resulting in continued inflammation through the release of inflammatory mediators. This cellular-driven late-phase response is seen over 4-8 hours and may persist for hours or days.

Patients that repeatedly come into contact intranasally with the allergen, tend to react with an immediate response on ever decreasing amounts of allergen (priming effect). Seasonal and perennial allergic rhinitis are discerned. Tree, grass, and weed pollens and outdoor fungal spores are common seasonal allergens. Dust mite faecal proteins, cockroach allergen, indoor fungi, cat, dog, and other danders, lead to persistent, perennial rhinitis. The determining factors in all these are proteins, mostly with protease activity (Matsumura, 2012).

The allergic sensitisation that characterises allergic rhinitis has a strong genetic component (Skoner, 2001).

3.3.2 Allergic asthma

This disease is a chronic inflammation of the airways resulting in increased contractibility of the surrounding smooth muscles (bronchospasm). The airways become inflamed and flooded with thick mucus. Common symptoms include wheezing, coughing, chest tightness, and shortness of breath. Asthma is caused by a combination of complex and incompletely understood environmental and genetic interactions.

Again, asthma can be divided in allergic and non-allergic types. Aeroallergens can include seasonal pollen, fungal spores, dust mites, animal allergens, and food.

Allergic asthma typically follows the type I response with APCs (*i.e.* macrophages, dendritic cells) in the lower airway capturing, processing, and presenting the antigen to helper T cells. The following cascade of processes leads to an inflammatory status eventually resulting in hypertrophy of smooth muscles, hyperplasia of mucous glands, thickening of basement membranes, and continuing cellular infiltration. These long-term changes of the airway (airway remodelling) may ultimately lead to fibrosis and irreversible airway obstruction in some patients.

Environmental factors may enhance the allergic reaction. Epithelial injury by viruses, bacteria, tobacco smoke, air pollutants and/or oxidative stress enhances allergen passage (Galli *et al.*, 2008).

3.3.3 Atopic dermatitis

Exposure to aeroallergens may also lead to atopic dermatitis (eczema) in predisposed individuals. It usually predates allergic rhinitis and asthma (Hogan *et al.*, 2012; O'Connell, 2004; Patrizi *et al.*, 2011). The causative agents are again animal dander, mites or dust, mould, and tree, grass, and weed pollen. In adult populations the relevance of atopic allergens diminishes with time.

3.3.4 Hypersensitivity pneumonitis (or extrinsic allergic alveolitis)

Hypersensitivity pneumonitis is an inflammation of the alveoli within the lung caused by inhaled organic dusts or fumes. The pathogenesis involves both type III hypersensitivity and type IV hypersensitivity reactions (Mohr, 2004). Fungi, bacteria, rat urine protein, mollusc shell dust, avian proteins (feathers and bird droppings), coffee bean protein (dust) etc. may be the responsible agents. More than 200 different organic antigens have been associated with the disease.

Hypersensitivity pneumonitis develops when exposed to high doses of soluble environmental antigens as in agricultural or industrial settings (Martel *et al.*, 2010).
The acute form typically starts with influenza-like symptoms 4-8 hours after exposure to the inciting antigen: cough, dyspnea, fever, chills, myalgias, and malaise. The peak of intensity occurs 12-24 hours later and symptoms resolve within 48 hours.
The subacute form results from repeated exposure to low doses (<4 months of exposure).
Chronic hypersensitivity pneumonitis evolves in pulmonary fibrosis developing over months to years in susceptible individuals after frequent or continuous exposure to the allergen (>4 months of exposure). The chronic form, with lung fibrosis, can be very severe, and even life-threatening.
However, hypersensitivity pneumonitis remains a rare disease, and is associated with very specific conditions and high level of exposure. A well-known example is the pigeon breeders lung.

3.3.5 Allergic laryngitis

Laryngitis generically refers to inflammation of the tissues of the larynx (throat). It may have several causes among which pathogens and airborne particles. Whether an allergic reaction can be leading to laryngitis is debated (Dworkin, 2008).

4. Individual predisposition

As only a fraction of individuals exposed to allergens develop allergy, this suggests that individual susceptibility factors play an important role in the expression of clinical disease. Susceptibility to allergic disease is a complex genetic trait with many polymorphic genes involved. A person's genetic constitution, variations in genes involved in immune/inflammatory regulation, can modulate how this person interacts with these agents. Many genes seem to be involved as it was found that many regions of the genome each account for only a small proportion of the disease phenotype (WAO, 2011). Examples of candidate genes are pattern recognition receptor genes, TGF-β1 gene, genes encoding inflammatory cytokines and enzymes, tumour necrosis factor-α gene, etc., involved in immune modulation.

Also a dysfunctioning epidermal barrier is an important factor (Hogan *et al.*, 2012; Patrizi *et al.*, 2011; WAO, 2011). While scratches or irritating chemicals may cause physical injury, also genetic factors may be involved. For example, in atopic individuals a mutation in the filaggrin (filament-aggregating protein) gene was found to be associated with atopic dermatitis (Patrizi *et al.*, 2011). This protein is involved in the epidermal barrier function. It is important for water permeability and for blocking the entry of microbes and allergens. A disruption of the skin barrier may lead in second instance to asthma and allergic rhinitis.

There are important gene-environment interactions, but the underlying mechanisms of susceptibility and variability in disease expression are not completely known at present.

5. Treatment

Allergic disease can usually not be cured. To date only insect venom allergy treatment can completely relieve symptoms, while immunotherapy with inhalant allergens usually provides partial protection. Allergen-specific immunotherapy (SIT) is time consuming and requires specialised care.
Avoidance is thus the first measure to take to prevent an allergic reaction. However, in practice this is not always possible. Accidental exposure is common and can cause an allergic reaction.

5.1 Pharmacotherapy

Pharmacological treatment is the mainstay of treatment to control symptoms (WAO, 2011). Most common medications have an antagonistic effect to block the action of allergic mediators, or to prevent activation of cells and degranulation processes. Antihistamines block histamine that is released by effector cells upon an allergic challenge, at the histamine receptor level. Glucocorticoids are part of the feedback mechanism in the immune system that decreases inflammation. Both antihistamines and glucocorticosteroids are available as intranasal applications for the treatment of allergic and non-allergic rhinitis. Yet, antihistamines cannot treat a severe allergic reaction.
Anti-leukotrienes are leukotriene receptor antagonists. Other possible medications are theophylline, cromones, anti-cholinergics, decongestants, mast cell stabilisers etc.

The recombinant DNA-derived humanised IgG1 monoclonal antibody, known as omalizumab, is effective to reduce sensitivity to inhaled allergens (allergic rhinitis, allergic asthma) and is studied for other allergy indications (Chang *et al.*, 2007). The antibody binds to free IgE and to membrane-bound IgE on B cells, but not to IgE bound by the high-affinity FcεRI receptors on basophils and mast cells or by the low-affinity FcεRII receptors on B cells. Omalizumab inhibits the binding of IgE to FcεRI on mast cells and basophils by binding to an antigenic epitope on IgE that overlaps with the site to which FcεRI binds. Consequently the release of the inflammatory chemical mediators by mast cells and basophils is reduced.

5.2 Treating anaphylaxis

For anaphylaxis, the airway, breathing, circulation, disability, exposure (ABCDE) approach is the correct emergency response (Working Group of the Resuscitation Council, 2008; WAO, 2011). The administration of intramuscular adrenaline (epinephrine) is the first-line treatment. Epinephrine is a hormone and a neurotransmitter regulating heart rate, blood vessel and air passage diameters amongst other functions. The α-receptor agonist function reverses peripheral vasodilation and reduces oedema. Its β-receptor activity widens the bronchial airways, increases the force of myocardial contraction, and suppresses histamine and leukotriene release (Working Group of the Resuscitation Council, 2008).
Individuals with a high risk of an anaphylactic reaction are advised to carry an adrenaline auto-injector and should receive training and support in its use (WAO, 2011). Intravenous administered antihistamine preparations and corticosteroids are used in conjunction with adrenaline but should not replace the latter.

5.3 Allergen-specific immunotherapy

For 100 years already SIT has been used as a desensitizing therapy for allergic diseases (Akdis & Akdis, 2011; Larché *et al.*, 2006; WAO, 2011).

The person is gradually vaccinated with progressively larger doses of the disease causing allergen. The aim is to achieve hyposensitisation in order to reduce the symptoms occurring during the natural exposure to the allergen. This can be done with an allergenic extract or vaccine, and in recent years with recombinant allergens. The beneficial effects of immunotherapy persist for years after discontinuation.

Several effects are observed in treated patients. Very early on the activity of basophils and mast cells is decreased, but little is known on the mechanisms (Akdis & Akdis, 2011). Treg cells play a pivotal role in inducing and maintaining immune tolerance. Following SIT generation of Treg cell secreting interleukin (IL)-10 and TGF-β is induced and the ratio of T_H1 cytokines to T_H2 cytokines is increased (Akdis & Akdis, 2011; Larché *et al.*, 2006; WAO, 2009). TGF-β might contribute to immunoglobulin class switching to IgA, IgG1 and IgG4 (Larché *et al.*, 2006). These immunoglobulins compete with IgE for allergen binding, decreasing the allergen capture and presentation that is facilitated by IgE.

For SIT still mostly natural allergens are used. Recently some synthetic peptides that contain T cell epitopes and recombinant allergens have been introduced. Using recombinant native allergens has advantages over natural extracts: the exact amount can be defined and the formulation can be standardised (Larché *et al.*, 2006; Valenta and Niederberger, 2007). Modified recombinant allergens may be even safer *e.g.* by making them hypoallergenic reducing the reactivity with IgE. Mutations, fragmentation of the protein or allowing for oligomer formation may alter the allergenicity while maintaining immunogenicity. Recombinant hybrid allergen proteins may combine epitopes of one or more allergens. The immunogenicity of the components is increased when assembled in one molecule, compared to separate molecules for each (Larché *et al.*, 2006; Linhart & Valenta, 2005). Synthetic peptides only containing allergen T cell epitopes are still able to immunomodulate T cells, but have difficulty to crosslink IgE and therefore minimise the allergy response.

The allergens are administered subcutaneously (subcutaneous immunotherapy, SCIT) or more recently orally (sublingual immunotherapy, SLIT). The latter method may be applied also in cases of food allergy and is said to be safer than SCIT having less side-effects (WAO, 2009). Also, patients undergoing immunotherapy may receive omalizumab, for the purpose of reducing the risk of anaphylactic reactions probably improving effectiveness.
In recent years it has been shown that “tolerisation” can be achieved in many cases of drug allergy. Since this is a rather complicated procedure and only offers a temporary effect, the treatment is only carried out in patients who are in urgent need of a specific medication such as in cancer patients (see Castells *et al.*, 2008).