# PROTOCOL FOR ALLERGENICITY TESTING OF GENETICALLY TRANSFORMED PRODUCTS IN ANIMAL MODEL

## INTRODUCTION

India is the 7th largest country in the world. Its population was 885 million in mid 1993 (16% of global population) which is expected to rise to 1015 million by 2000 AD and 2530 million by 2050 AD. It is absolutely necessary to increase the food production to feed the growing population. Although the food production has increased but it was almost proportional to the rise in the population with the result the per capita consumption remained almost static during the last one decade. Therefore, in order to further improve the quality and quantity of agricultural crops, various means are to be adopted. India has realised that there is no escape from the use of modern biotechnology involving the application of genetically modified plants to accomplish increased food production. Modern biotechnology includes genetically modified agricultural crops or foods, modified through human intervention by recombinant DNA technology. However, the unfamiliarity with these new inventions had aroused great concern among regulatory agencies for the right assessment of risk from the use of genetically modified plants to the habitat including human and animals. This situation is true, not only in India but also in all the developing countries as well as in the developed nations (Ghosh, 1997).

It is known that almost any food may be responsible for allergic disorders. Therefore, it is important and also would be appropriate not to overlook the allergenic potentials of genetically derived foodstuffs. For foods or food components produced by biotechnology, the safety assessment is particularly important (FAO/WHO, 1996). Unfortunately, model experimental protocols have not yet been designed to assess the allergenicity of new genetically modified foods. The tests to assess allergenicity are required in the case of transgenic crops, since exogenous proteins are engineered (Dean, et.al., 1996; McClintock, et.al., 1995; Niestiji, et.al., 1994; Taylor, 1985). To minimise the risk of allergenicity in exposed population, it is essentially required to identify whether the allergenic proteins are present or not, in the genetically engineered crop (Taylor, 1997). The assessment of allergenic potentials must be conducted in a careful step wise manner by using the in-vivo and in-vitro immunological tests.

A protocol is therefore, proposed by designing appropriate experiments to gather data on allergenicity testing in laboratory animals, which may help to evaluate the allergic potentials of new foodstuffs and thereby minimising the risk of allergenicity in exposed human population.

### *Brief Background of Allergy*

Food allergy appears to be relatively common in the community. In taking the past histories of atopic patients, acute episodes of urticaria, angioedema, itching or

gastrointestinal disorders following the ingestion of certain foods have been reported by substantial number of patients. These manifestations of hypersensitivity to foods are well known.

Allergic reactions occur when an already sensitised indiviual is re-exposed to the same foreign substance or allergen. Allergic responses range from the familiar runing nose and sneezing to systemic anaphylaxis and death. These responses do not occur when a naive individual is first exposed to an allergen. The initial response takes time and usually does not cause any symptoms. once an individual is sensitised, the allergic reactions often become worse with each exposure, as each re-exposure not only produces allergic symptoms but also increases the level of antibody present.

There are two distinct types of allergic reactions to foods, one is characterised by the rapid appearance of symptoms, often within a few minutes after the offending food is eaten and the second is delayed type in which a number of hours or even a day or more may elapse between the ingestion of the allergenic food and the appearance of symptoms. It has been suggested that the actual allergen in the case of the immediate type of reactions to foods are the whole proteins. With delayed reactions, the allergen may be some protein breakdown product formed during the process of digestion of the protein (Goldman, et.al., 1963).

The first description of the mechanism of allergic reaction was presented by Prausnitz and Kustner in 1921, which is similar to the PCA test used for the assay of IgE production in experimental animals. Prausnitz and Kustner proposed the existence of an ``atopic reagin'' in the serum of allergic subjects. Some 45 yrs. later, Ishizaka and colleagues isolated this atopic reagin and showed that it was a new class of immunoglobulin IgE. It is the presence of such highly specific antibodies which provide the basis for detecting the specific allergen to which the patient reacts. The level of specific IgG has little correlation to symptoms of food allergy. Other immunoglobulins (IgA, IgM & IgD) play no known role in allergic disorders.

***Animal Model***

Unfortunately, no established animal model is available in literature for assessing the allergenicity of genetically modified foods, however, rabbit or guinea pig could be used to detect allergenicity. Brown Norway rats (Atkinson and Miller, 1994; Atkinson, et.al. 1996) have been used in experimental studies but it is yet to be accepted as an animal model by the regulatory agencies. However, this could be used to generate additional data.

***Treatment Schedule***

Normal adult healthy animals are kept under proper husbandry conditions with 12 hour light and 12 hour dark period. Before the start of experiment, animals are kept one week for acclimation. Animals are randomly divided into two groups, based on diet fed. Experimental group consists of ten animals, sensitised to the test protein(s) by incorporating into the feeding pellets such that 10% of the total diet is from the test compound. The control group of ten animals are fed 10% of the total diet of non-transgenic foodstuff. Animals are fed for sixty days. Water is provided *ad libitum*. Sera from the treated animals are used to assess allergenicity.

### *Preparation of Antigen/Allergen*

Collect the test material in as pure form as possible, grind into fine powder form and defat with ether. Extract the defvatted material with buffered saline, 2% wt./vol/., however, this proportion can be varied depending upon the type of test compound. Allow the mixture to stand at 20*c for 72 hr., shake during this period 8-10 times for 30 minute each. Filter through Whatman No. 1 filter paper. Dialyse through dialysis sac, sterilise by passing through memberane filter, transfer to sterile vials and lyophilise and seal the vials.

### *Experimental Protocol*

The following *in-vivo* and *in-vitro* immunological assays could be used for the detection of reactogenic antibodies in the test sera:

## IN-VIVO ASSAYS

### 1. Passive Cutaneous Anaphylaxis (PCA)

### *Application and Limitations of Test*

PCA is an *in-vivo* method usually employed to assay the specific IgE present in serum. It is a useful immunological tool to detect as little as 0.1 ug antibody protein. In this test the anaphylactic reaction is visualised as a local skin reaction.

### *Sex of Animals*

Male and/or female healthy young adult animals can be used. if females are used they should be nulliparous and non-pregnant.

### *Housing and feeding Conditions*

Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. If guinea pigs are used, it is essential that animals receive an adequate amount of ascorbic acid.

### *Preparation of the Animals*

Animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by close clipping, shaving or by chemical depilation. Care should be taken to avoid adbarding the skin.

### *Principle*

PCA could be produced with the sera of allergic host by challenging intradermally sensities sites with intravenously injected antigen/allergen plus dye. Well-defined blue areas appear, indicating the sites of antigen-induced extravasation of fluid due to interaction with tissue fixed antibody.

***Description of Test Procedure***

Naive animals are shaved on the back and flanks, avoiding abrasion of skin. Unblemished skin sites are selected and cleaned with 70% alcohol. Injections are spaced approximately 3-4 cm apart.

0.1 ml of test serum is injected intradermally. Doses are usually in the ratio of 1 : 2 : 4 or 1 : 3 : 9. The doses are injected using tuberculin syringe(s). The syringe used for intradermal injection must not leak even under heavy injection pressure.

Plastic disposable sterilised tuberculin syringes are satisfactory in this respect. Glass tuberculin syringes must be checked for leaks both at the needle butt and past the piston. Needles with `short bevel' points, usually 1/2 inch x 26 gauge are used 24 to 48 hour later, 0.6 ml of antigen/allergen (1 mg/ml) is injected intravenously together with 0.4 ml of Evans blue (2% in physiological saline). For the intravenous injection the piston of the syringe must be easy moving so that there is no doubt that the needle is in the vein when the smallest pressure is applied. The needle must be very sharp. For intravenous administration in guinea pigs, the vein which runs on the dorsal surface of the hind foot between the metatarsals of the outer and middle toes or ear vein or intracardiac route can be used. In rabbits, the vein running the margin of ear is the most useful site but other veins which are easily accessible can also be used.

***Observation***

30-45 min. later, animals are killed. The skin is opened and reflected so that the lesions can be evaluated. Measurements of diameter and the assessment of intensity are usually made. This can be postponed until all the animals have been killed but the delay is not advised and should not exceed 2 hours. The skin must not be allowed to dry. Intensity of bluing is often expressed arbitrarily as + to +++. Since the relationship between area of response and the dose is roughly linear, the plot of mean diameter upon log dose will also be linear. A table showing both would usually be preferred.

When the potency of sensitising antibody is unknown, a wider range of doses may be useful . The highest dose should give lesions of about 15 to 20 mm. diameter and the smallest about 5 mm.

***Report on PCA***

Test Animal :

Sex :

Test Sample :

Date of Experiment Started :

Date of Experiment Terminated :

**Report of PCA :**

| Group | | Animal No. | Area of Dye extravasion | | |
|---|---|---|---|---|---|
| | | | Undiluted | 1:2 diluted | 1:4 diluted |
| | | | Sera | Sera | Sera |
| | | | | | |
| Experimental | | 1 | | | |
| | | to | | | |
| | | 10 | | | |
| | | | | | |
| | | | | | |
| Control | | 11 | | | |
| | | to | | | |
| | | 20 | | | |

**2. Prausnitz - Kustner (PK) Test**

***Application and Limitations of Test***

PK test is a reasonably accurate biological test when determining reaginic potency by wheal size. It's coefficient of variation is 20-30 per cent. The minimum dose of IgE antibody to give PK reaction has been estimated as 1 - 100 pg. Before testing the sera in a naive animal, it should be ascertained that the naive animal should not have any reactivity to the test antigen/allergen.

***Principle***

When normal skin is injected with reaginic serum, the reaginic antibodies become

attached to the skin mast cells and the injected area of skin acquires the specific skin reactivity towards antigen/allergen challenged.

***Sex of Animals***

Male and/or female healthy young adult animals can be used. If females are used they should be nulliparous and non-pregnant.

***Housing and Feeding Conditions***

Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. If guinea pigs are used, it is essential that animals receive an adequate amount of ascorbic acid.

***Preparation of the Animals***

Animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by close clipping, shaving or by chemical depilation. Care should be taken to avoid avrading the skin.

***Description of Test Procedure***

Intradermal injections are spaced approximately 3 - 4 cm apart. Unblemished skin sites are selected and cleaned with 70 % alcohol. The syrings used for the intradermal injections must not leak under injection pressure. Plastic disposable sterilised tuberculin syrings are satisfactory in this respect but the calibration at 0.05 ml must be checked.

If glass tuberculin syringes are used, they must be checked for leaks both at the needle butt and past the piston.

0.05 ml of serum (or a dilution of it) is then injected intradermally in the indicated position. Control site is injected with 0.05 ml of physiological saline. Care is taken to inject as far as possible always to the same depth of skin. The resulting injection wheals are then carefully outlined with a black or blue felt pen or ball-point per or otherwise marked so as not to rub off easily. 24 to 48 hour later, the experimental and control sites are challenged with 0.5 ml antigen/allergen.

***Observation***

A wheal and flare formation (> 3 mm) in the skin within 20 - 30 min. indicate positive reaction. The reaction wheals are outlined with a ball-point pen. Control site should not give wheal and flare response (Prausnitz and Kustner, 1921).

***Report on PK Test***

Test Animal :

Sex :

Test Sample :

Date of Experiment Started :

Date of Experiment Terminated :

**Report on PK Reaction**

| Animal Number | Group | Wheal & Flare response |
|---|---|---|
| | | |
| | | |
| | Experimental | |
| 1 | | |
| to | | |
| 10 | | |
| | | |
| | Control | |
| 11 | | |
| to | | |
| 20 | | |
| | | |

**IN-VITRO ASSAYS**

**3. Radioallergosorbent (RAST)/RAST Inhibition Test**

***Application and Limitations of Test***

It is considered to be a highly sensitive and allergen specific of all the currently available laboratory tests. RAST is a direct assay method where only the radioactivity is measured. In this test a smll increase of radioactivity above background becomes significant and it is this that gives the method its great sensitivity. 10 pg of IgE being quoted as the lower limit of sensitivity.

***Principle***

The antigen/allergen coupled to an insoluble polymer is added to the serum to be investigated, if the antibodies to the antigen/allergen are present, they react with the conjugate. After the removal of all unbound serum components, 125-I labelled anti-IgE antibodies are added. They will bind to the antibodies of the IgE class which have reacted with the polymer-coupled antigen/allergen. The uptake of labelled antibodies measured in terms of radioactivity is proportional to the amount of IgE antibodies (Wide, et.al., 1967).

***Description of Test Procedure***

Insoluble Polumer-allergen conjugate : The allergen in 1 ml solution is coulpled to 100 mg CNBr-activated Sephadex G 25 or paper discs. The particles/discs are then suspended in a concentration of 1 mg/ml of 0.1 M tris buffered saline solution of pH 7.4 with 1% Tween 20 and 0.2% bovine serum albumin. The conjugates are stable for atleast 3 months at + 4°C.

125-I labelled anti-IgE antibodies : The purified antibodies labelled with 125-I can be purchased from the market or may be labelled with 125-I using the chloramine - T.

5-50 ul of serum and 0.5 ml of suspension of polymer-allergen conjugate are mixed in a test tube and incubated for 6-24 hour with low vertical rotation. The suspension is centrifuged at 3000 rpm and washed three times with tris buffer saline solution of pH 7.4 containing 1% tween-20. 100 ul of labelled anti-IgF antibodies in a concentration corresponding to about 40,000 cpm is added to the tubes. The mixture is incubated, centrifuged and washed. The bound radioactivity is measured in Scintillation counter. The results obtained with unknown sera were compared with known non-allergic sera and with diluent.

In Rast inhibition, allergen is added to serum from a host sensitive to this allergen. An inhibition of reaction takes place in RAST system when tested for antibodies to this particular allergen.

***Standard curve***

The standard curve or dose response curve is obtained by using various concentrations of standard. It is preferable to use as many standard concentrations as possible (10-12 concentrations) but they should not be less than five. It is better to include serial double dilutions of standards but where the working range of standard curve is small, other intermediate dilutions can also be included to increase the number of standards in the assay.

***Observations***

Ths results are regarded as positive (+) when the radioactivity uptake is 2 - 5 times that of the control and strongly positive (++) when the radioactivity is higher. All tests are conducted in duplicate.

RAST Score = cpm in test sera (mean of duplicate)/cpm in known negative (mean of duplicate)

Score of two or greater indicates allergen specific IgE.

***Report on RAST***

Test Animal :

Sex :

Test Sample :

Date of Experiment Started :

Date of Experiment Terminated :

| Animal Number | Group | Counts per minute (cpm) |
|---|---|---|
| | | |
| | | |
| | Experimental | |
| 1 | | |
| to | | |
| 10 | | |
| | | |
| | Control | |
| 11 | | |
| to | | |
| 20 | | |
| | | |

**4. Enzyme-Linked Immunosorbent Assay (ELISA)**

***Application and Limitations of Test***

ELISA is an *in-vitro* assay of high sensitivity, close to solid phase radioimmuno assay. It is a relatively simple test which does not require special equipments and uses antibodies which remain stable for long periods under frozen conditions. IgE levels ranging from 24 - 24000 ng/ml are found in human sera and correlate well with RAST results. Reproducibility is said to be as high as ±3% and it is claimed that as little as 10 ng IgE/ml can be detected and that the test may be completed within 24 hour. The importance

of high quality anti-IgE antibodies is stressed and it is also pointed out that less potent antibodies may require higher temperature and longer incubation periods to obtain maximum accuracy.

***Principle***

The IgE under test is made to react with solid phase anti-IgE. In ELISA, an enzyme is used in place of 125-I. The label taken up by the washed solid phase is proportional to the IgE content of the sample under test and is measured spectrophotometrically.

***Description of Test Procedure***

The test is conducted by adsorption or coating of wells of microtitre plates with the test proteins. Unadsorbed proteins are removed by three washings. Test sera is incubated in the washed plates. The plates are again washed followed by incubation with enzyme labelled anti-IgE antibodies. Substrate is added in the washed plates, to produce colour. Optical density (OD) of colour is measured in automatic scanner or ELISA plate reader, which is directly proportional to the concentration of IgE antibodies (Vos, et.al., 1979). (If protein extract of the sample is unable to coat the wells, in that case the assay may be conducted for the estimation of total IgE, yet the estimation of specific IgE is essential as total IgE will not reflect allergenicity).

***Observations***

Blank values, i.e. those obtained from the wells which were not coated with antigen/ allergen are subtracted from the test values. Test is considered positive when the values are two fold or more higher than the controls. Each plate must contain positive and negative controls. Each sera must be tested in duplicate or triplicate wells.

**Report on ELISA**

Test Animal :

Sex :

Test Sample :

Date of Experiment Started :

Date of Experiment Terminated :

| Group | | Animal No. | | Optical Density | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Dilutions of Sera | | | | |
| | | | | 2 $\log^2$ | 2 $\log^3$ | 2 $\log^4$ | 2 $\log^5$ | to 2 log |
| | | | | | | | | |
| | | | | | | | | |
| Experimental | | 1 | | | | | | |
| | | to | | | | | | |
| | | 10 | | | | | | |
| | | | | | | | | |
| | | | | | | | | |
| Control | | 11 | | | | | | |
| | | to | | | | | | |
| | | 20 | | | | | | |
| | | | | | | | | |

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# ADDENDUM TO THE "REVISED GUIDELINES" - AUGUST, 1998

Dated: 24th September, 1999.

1. Please add the following **'sub-para xi'** after the 'sub-para x' of para **B. RCGM (Review Committee on Genetic manipulation)** at page 4 of the "Revised guidelines", which reads as under:

**Sub-para xi :** RCGM can approve applications for generating research information on transgenic plants. Such information may be generated in contained green house as well as in very small plots, as research needs to be conducted in such environment for seeking answers to specific environmental safety issues emanating from the use of transgenic plants. The small experimental field trials should be limited to a total area of 20 acres in multi-locations in one crop season. In one location where the experiment is conducted with transgenic plants, the land used should not be more than one acre. Any experiment beyond the above limits in one crop season would require the approval of the Genetic Engineering Approval Committee (GEAC).

2. Instead of the following caption of "Appendix III" at page 18 of the "Revised Guidelines....."

"Application seeking Non-Regulated Status in India under the Environment (Protection) Act-1986 for Transgenic Plants".

please read as:

*"Application seeking Approval under Rules 8,9,10 & 11 of the Notification No, GSR 1037(E) dated 05.12.1989 issued by the Ministry of Environment & Forests under the Environment (Protection) Act, 1986, for transgenic plants".*

3. Instead of the following caption on page 21 of the "Revised Guidelines....."

"Particulars of the application seeking Non-Regulated status under Environment (Protection) Act-1986".

please read as:

*"Particulars of the application seeking Approval under Rules 8,9,10 & 11 of the Notification No, GSR 1037(E) dated 05.12.1989 issued by the Ministry of Environment & Forests under the Environment (Protection) Act, 1986, for transgenic plants".*