ATTACHMENT 6

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Monsanto Company
Biotech Regulatory Science
Final Report

REPORT NO.: MSL-15740

DATE: 11/18/ 98

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AUTHORS: **Richard E. Goodman and Larry R. Holden**

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Study Number: 98-01-36-01

Title: **Assessment of the Allergenicity of Bollgard Cottonseed Proteins Relative to Conventional Cottonseed Proteins.**

Study Authors: Richard E. Goodman
Larry R. Holden

Contributors: John Leach, Joan Lee, Dave Harah

Record Retention: All study specific raw data, protocols and final reports will be retained at Monsanto, St. Louis, except the raw data developed at Ralston Analytical Labs, for analysis of the raw cottonseed meal, which is retained at the Ralston Analytical Laboratory, 824 Gratiot, St. Louis, MO 63102. Actual dietary composition, other than the cottonseed, is proprietary and is held at the Richmond, IN facility of Purina TestDiet, 1050 Progress Dr., Richmond, IN 47374.

Sample Retention: Any study samples which are to be retained will be stored at Monsanto, St. Louis.

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ABBREVIATIONS

ACA	Active cutaneous anaphylaxis
BN	Brown Norway rats
Bt	*Bacillus thuringiensis* insect-resistance gene
cry1Ac	Class I (Lepidopteran-specific) crystal protein gene
Cry1Ac	Class I (Lepidopteran-specific) crystal protein from *Bacillus thuringiensis* var. *kurstaki*
CSM	cottonseed meal
DHL	DHL Worldwide Express
FASTA	DNA sequence homology computer program
g	gram
mg	milligram
µg	microgram
ml	milliliter
µl	microliter
FcεRI	IgE high affinity receptor
i.p.	intraperitoneal
IgE	antibody isotype IgE
MAHYCO	Maharashtra Hybrids Seeds CO. Ltd.
MECH-1	MAHYCO cotton hybrid #1
MECH-3	MAHYCO cotton hybrid #3
MECH-12	MAHYCO cotton hybrid #12
MECH-15	MAHYCO cotton hybrid #15
MECH-915	MAHYCO cotton hybrid #915
nptII	neomycin resistance, selectable marker gene (Neomycin phosphotransferase II gene)
NPTII	neomycin resistance protein (amino-glycoside-3'-phosphotransferase II)
SAS®	SAS® statistical analysis software
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
w/v	weight to volume

1. SUMMARY

This animal model study was designed to compare the relative reaginic activity, or allergenicity, of cottonseed from genetically modified hybrid cottonseed containing the insect resistance gene *cry1Ac* and selectable marker gene *nptII*, with cottonseed from three non-transgenic hybrids. The high IgE responder Brown Norway rat was used as the test system. Approximately 6 week-old rats were fed diets containing no cottonseed meal, or diets with non-transgenic cottonseed meal (MECH-1) included at a rate so that 5% or 10% of the protein in the diet was from cottonseed. All animals gained weight at similar rates and remained clinically symptom free throughout the study. After 62 days of feeding, each animal was challenged by intradermal injection of four concentrations of crude cottonseed protein extract from the transgenic hybrid (MECH-1 Bt) and three non-transgenic hybrids (MECH-1, MECH-3, MECH-12). Systemic injection of dilute Evans blue dye provided a visible mark of areas of local extravasation at dermal challenge sites as an indicator of immune or inflammatory reactivity. Based on statistical analysis of the diameters of blue spots and direct observation, the reaginic activity (allergenicity) and inflammatory characteristics of transgenic cottonseed (MECH-1 Bt) were equal to those of the corresponding non-transgenic (MECH-1) and were clearly within the range of activities determined for two other non-transgenic cotton hybrids. These data lead to the conclusion that there is no significant biological difference between the reaginic reactivity (allergenicity) of the cottonseed meal from the transgenic and the three commercial non-transgenic cotton hybrids.

2. INTRODUCTION

Monsanto Company has developed Bollgard™ (a registered trademark of Monsanto Co.) cotton containing the *cry1Ac* gene, encoding a Lepidopteran specific insecticidal protein, Cry1Ac that was originally isolated from *Bacillus thuringiensis* var. *kurstaki*. The *cry1Ac* gene was transferred into Bollgard cotton along with the marker gene, *nptII*, which expresses the neomycin phosphotransferase II (NPTII) protein. Monsanto Company and Maharashtra Hybrids Seeds CO. Ltd. (MAHYCO) have crossed Bollgard cotton with Indian cotton (MECH-1) to incorporate the *cry1Ac* gene into Indian cotton hybrids.

2.1 Background

Historically, various *Bacillus thuringiensis* formulations have been safely used for more than thirty years to kill insect pests in a variety of crops, ornamentals and trees (reviewed in Entwistle et al., 1993). As literature searches indicate, this use has not led to any documented cases of allergic

responses to the insecticidal proteins. Cry1Ac is insecticidal to lepidopteran larvae that ingest the proteins. It acts by binding to a lepidopteran larval specific mid-gut membrane receptor and after activation in the highly basic gut fluid, induces perforation of the gut. This protein has been shown to have no adverse biological effects in non-target species including mammals.

In a previous study by Monsanto Company (Astwood, 1995a), the amino acid sequence of the Cry1Ac protein (HD-73) was compared with sequences of known allergens using the FASTA algorithm (Pearson and Lipman, 1988) to determine if there are any significant regions of sequence homology (8 or more identical amino acids). Homology of this nature would indicate an increased likelihood that the protein might be recognized by antigen specific IgE of allergic individuals and possibly would act as an allergen in those individuals having a specific allergy. No such homology was identified.

Gastric stability, as measured by *in vitro* digestibility assays with pepsin-HCl, has been shown to correlate with food proteins that are allergenic. Proteins that are unstable in the assay are unlikely to be food allergens (Metcalfe et al., 1996). An *in vitro* assessment of the digestibility of Cry1Ac performed previously at Monsanto (Ream, 1994) showed that the Cry1Ac protein was rapidly degraded in simulated gastric fluid as measured by the loss of activity in bioassay (tobacco budworm mortality) and by loss of binding in antigen specific western blot analysis.

Gastric stability and animal safety studies have also been performed with the NPTII protein. This protein was degraded rapidly in the *in vitro* gastric digestion model and was determined to be safe in oral animal dosing (Ream, 1993; Fuchs et al., 1993). In addition, no homology to known allergens was identified (Astwood, 1995b).

There is no evidence in the literature and the cited studies that would implicate either Cry1Ac or NPTII proteins as allergens.

Cottonseed meal or meal fractions are rarely consumed by humans because they contain gossypol, a polyphenolic compound that is moderately toxic to various monogastric species, and relatively non-toxic to ruminants once the rumen is established and functional (Abraham and Hron, 1992). However, previous human consumption has led to the conclusion that commonly grown, non-transgenic cotton seeds contain at least one endogenous protein that has been identified as causing serious human allergic disease if ingested directly by sensitized individuals in the form of baked bread containing cottonseed

flour or commercially produced health-food energy bars (Bernton et al., 1940; Spies et al., 1953; Atkins et al., 1988; Malanin and Kalimo, 1988). Allergic reactions to cottonseed products have been restricted to the protein-containing fractions and have not been associated with refined cottonseed oil products (Bernton et al., 1940; Figley, 1949).

Prior to commercialization of Bollgard cotton in India, studies were undertaken to demonstrate the safety of Indian hybrid lines of Bollgard cottonseed proteins and meal relative to typical parental hybrid lines of Indian cotton. This report describes and summarizes a study which was performed in a Brown Norway rat, to compare the reaginic (i.e., antibody mediated allergic response) activity of Bollgard hybrid cottonseed proteins relative to proteins from commercial non-transgenic cotton hybrid seeds. Whereas it is recognized that there is no animal model that has been validated to predict allergenicity in humans, the Brown Norway rat has been used to measure reaginic antibody responses to various allergenic and non-allergenic food proteins, and as a model for testing various mechanistic developments of the immune response (Knippels et al., 1998; Ju et al., 1995; Atkinson and Miller, 1994; Diaz-Sanchez and Kemeny, 1991; Stewart and Holt, 1987).

The results of this study lead to the conclusion that transgenic Bollgard cottonseeds are no more allergenic than non-transgenic cottonseeds. The current study was performed in response to the request from the Indian Department of Biotechnology to provide further evidence, from an animal model, as to whether transgenic cottonseed might be more allergenic than non-transgenic cottonseed.

2.2 Purpose

The purpose of this study was to assess the relative allergenicity of Bollgard cottonseed proteins compared to conventional cottonseed proteins, as measured by active cutaneous anaphylaxis (ACA) in cottonseed-fed Brown Norway rats. The comparison assesses whether the allergenicity of the protein components of conventional cottonseed have been altered by the introduction of the insect resistant gene, *cry1Ac*, or the selectable marker gene, *nptII* into Bollgard cotton as measured by the binding of reaginic antibodies in cottonseed sensitized Brown Norway rats.

3. EXPERIMENTAL DESIGN

Prior to the initiation of this study, Study Protocol # 98-01-36-01 (Appendix 7.1) was prepared outlining the design and expected conduct for this study.

The overall design was to sensitize Brown Norway rats to cottonseed proteins by feeding cottonseed meal or control diets, then testing by *in vitro* and *in vivo* methods for the production of antigen specific IgE. At the beginning of the study, the study design was changed to concentrate on the *in vivo* ACA assay as the best method to analyze the reaginic immune response in a biologically relevant test system (Appendix 7.1.1). Five animals were assigned to each feeding group (no cottonseed control, 5% cottonseed meal, and 10% cottonseed meal diets). At the end of the study, each animal was identically challenged by 16 intradermal injections of cottonseed meal (four hybrid cottonseed extracts at four concentrations each, Table I). Skin reaction sizes were scored for comparison between hybrid extracts, doses and diets. This design allows direct comparison of each cottonseed test extract material while reducing the influence of animal to animal variation.

Table I. ACA cottonseed challenge extract injection doses for all animals. All animals were tested on the same day, with the same diluted extracts.

Dose\Extract	MECH-1	MECH-1 Bt	MECH-3	MECH-12
0.31 µg	X	X	X	X
1.25 µg	X	X	X	X
5.00 µg	X	X	X	X
20.0 µg	X	X	X	X

3.1 Materials

a. Cottonseed. Seed of transgenic cotton (MECH-1 Bt) and non-transgenic cotton (MECH-1, MECH-3, MECH-12, MECH-15 and MECH-915) were harvested, acid delinted by MAHYCO and shipped via DHL, through U.S. Customs to Monsanto Company. Proximate analysis was conducted on ground, whole cottonseed meal of all six hybrids. Raw, ground seed of non-transgenic MECH-1 was the only hybrid used in the manufacture of the feed for the rats. Samples of all hybrid seed meals were extracted with hexane to remove lipids prior to extracting proteins in aqueous solution for characterization with *in vitro* tests (protein analysis and SDS-PAGE and Cry1Ac immunoassay), and to use as challenge doses in ACA assay. The *in vitro* characteristics of MECH-15 and MECH-915 were similar to those of the other hybrids and these were not used in the ACA assay. Protein extracts of MECH-1, MECH-3 and MECH-12 were compared with an extract of equally treated MECH-1 Bt in the *in vivo* ACA assay.

b. Raw cottonseed meal. Cold (-20°C) delinted cottonseed lots were weighed and ground to a moderately fine powder with dry ice (CO_2) pellets, using a commercial/industrial Waring blender. Powdered meals were stored frozen (-20°C), with permeable covers and mixed occasionally over two days to allow the CO_2 to sublimate from the meal. The ground cottonseed meals were maintained frozen (-20°C).

c. Defatted cottonseed meal. Samples for protein gel electrophoresis and for challenge dosing in the active cutaneous anaphylaxis (ACA) assays were defatted by three serial extractions of each ground meal in six volumes of hexane (HPLC, UV spectrophotometry grade) at 45°C to 50°C with mixing for 15 minutes. The hexane was removed by filtration after each extraction and residual hexane was removed by evaporation at room temperature under vacuum.

d. Cottonseed meal analysis. Samples of raw cottonseed meal were submitted to Ralston Analytical Labs for analysis of gossypol content (MECH-1 and MECH-1 Bt only) and for proximate analysis (all hybrids).

e. Rat diet pellet formulation. Dr. Dorrance Haught, primary animal nutritionist of Purina TestDiet in St. Louis, MO, formulated three diets based on standard, commercial Purina Laboratory Rodent Diet 5001. These were a control diet containing no cottonseed meal and two feed sensitization diets containing full-fat, ground, raw, non-transgenic MECH-1 cottonseed. The percentage of cottonseed in the cottonseed containing diets included sufficient meal such that 5% or 10% of the protein in the diet was from the cottonseed meal. All three diets were balanced with approximately equal composition of total protein, lipid, carbohydrate and fiber. The other major plant material sources were soybeans, corn, oats, sugar beet, wheat and alfalfa. The diets contained fish meal and domestic animal meat. The exact proportion of components in the diets is proprietary (Purina) except for the concentration of cottonseed meal.

f. Rat diet pellet manufacture. The three test diets were manufactured in successive batches at Purina TestDiet, Richmond, IN, USA, following the specifications developed by Dr. Haught.

g. Protein extracts for intradermal challenges. Defatted cottonseed meal was extracted for two hours with mixing at room temperature in sterile saline (0.9%) with 1 g meal plus 10 ml saline. Insoluble material was removed by centrifugation and solutions were sterile filtered. Aliquots of

extracts were frozen at -80°C and thawed at room temperature immediately prior to dilution and use. Protein concentrations of samples were determined by a commercial Bradford dye binding assay (BioRad; Emeryville, CA). As an additional ACA control, non-transgenic commercial soybeans were ground, the meal was defatted and extracted by the same method as the cottonseed meal. Prior to intradermal injection in the ACA assay, sample aliquots were serially diluted with sterile saline to the expected concentrations. Equal concentrations of cottonseed extracts were qualitatively evaluated by SDS-PAGE gel (10-20% acrylamide, tris-glycine, Novex; San Diego, CA) electrophoresis of reduced samples (heated at 95°C for five minutes in Laemmli buffer).

h. Evans blue intracardiac dose. Evans blue dye crystals (Sigma Chemical; St. Louis, MO) were dissolved in sterile saline (0.9%) to a final concentration of 1% (w/v) just prior to injecting during the ACA assay.

i. Histamine intradermal positive control. Histamine base (Histatrol), at 1 mg/ml in 50% glycerol from Center Laboratories, Port Washington, NY, was used as a positive control for the Evans blue injection in the ACA assay.

j. Pentobarbital anesthetic. Sterile pentobarbital (65 mg/ml) was diluted 1:4 with sterile saline and used by injecting animals i.p. prior to performing the ACA. Injection doses were administered at 32.5 mg/kg body weight, with additional boosting if necessary at approximately one half that dose.

k. Cry1Ac detection assay. An immuno-detection assay was used to detect the presence of Cry1Ac protein in seed samples. These tests used a Cry1Ac specific monoclonal antibody and detection system. This assay was used to qualitatively demonstrate that the MECH-1 Bt test materials contained the transgenic protein and that the non-transgenic materials did not contain the Cry1Ac protein.

3.2 Animals

Brown Norway Rats. Nulliparous, non-pregnant female Brown Norway rats between 75 g and 100 g total body weight (approximately 6 weeks of age) were purchased from Charles Rivers Laboratories, Raleigh, NC facility. Animals arrived March 24, 1998, were acclimated on control diets with food and deionized water provided *ad libitum*. Animals were arbitrarily assigned to treatment groups, marked with uniquely numbered metal ear-tags and housed with 3 to 5 animals per cage, in polycarbonate suspended cages with commercial, ground corncob bedding. Five rats were assigned to each of the

three feeding groups and were subsequently used in the ACA assay. One of the animals (rat # 63) died from anesthetic overdose during the ACA challenge and was replaced by an equally treated rat from a parallel experiment (Appendix 7.1.2). Body weight data for the replacement rat (#71) was used instead of the data from rat #63 in the analysis reported here.

3.3 Feeding Exposure

All rats were started on study diets March 31, 1998, fed *ad libitum* and maintained on the appropriate study diet throughout the 62-day period. All animals were observed daily and weighed approximately weekly. Cages and bedding were changed biweekly. Daily care was provided by the Monsanto Animal Research Facility in St. Louis, MO, which is accredited through the American Association for Accreditation of Laboratory Animal Care.

3.4 Active Cutaneous Anaphylaxis (ACA)

ACA is a direct *in vivo* immunoassay designed to challenge sensitized animals with potentially allergenic materials (Inagaki et al., 1992; Befus et al., 1982; Church, 1975). As designed in this study, animals were sensitized by feeding the test materials (raw cottonseed meal) for 62 days. After sensitization, the animals were challenged by intradermal injection of various extracts of cottonseed extract. If antigen specific IgE was induced during sensitization, the IgE would bind antigen during challenge the complex and cross-link IgE receptors (FcεRI) on tissue mast cells. The cross-linking would cause degranulation of the Mast cells, releasing inflammatory mediators including histamine and leukotrienes. The mediators act on local capillary beds, causing local vascular leakage and allowing blue dye to escape into the tissue. The volume of leakage, and the area of stained tissue should correlate with the concentration of specific IgE and the dose of the challenge antigen.

In this study design fifteen anesthetized, depilated rats (five from each dietary feeding group) were administered intradermal injections of dilute solutions (50 µl) of protein extracts containing 20 µg, 5 µg, 1.25 µg or 0.31 µg of total protein from four cotton extracts (MECH-1 Bt, and the non-transgenic commercial lines MECH-1, MECH-3 and MECH-12). The intradermal injections were given in quick succession in the pattern illustrated in Appendix 7.5. Injections were administered in the dorsal trunk region, with a 27 gauge hypodermic needle. By injecting each rat with each extract and each dose, the comparisons minimized inter-animal variation. An extract of commercial, non-transgenic soybean meal was prepared similar to the cottonseed meal and was injected in duplicate at a dose of 5 µg total

protein. The purpose of the soy extract was to test the ACA response to a typical dietary protein source. Fifteen to thirty minutes after injecting the challenge doses, a saline solution (1 to 2 ml) containing 1% Evans blue was injected in the right ventricular chamber of the heart. Approximately ten minutes later, a positive control dose of histamine containing 10 µg free base, was injected at two sites. The animals were killed by asphyxiation with CO_2, or lethal injection of pentobarbital 10 to 20 minutes later. Skins were removed, inverted and the diameter of the blue spots marking the area of immune or inflammatory mediated vascular leakage were measured and recorded. In these experiments, photographs were also taken to record the spot pattern (Appendix 7.5).

3.5 Statistical Methods

Analyses were performed using SAS® (SAS Institute Inc., Cary, NC). Analysis of the body weight data was performed using a one-way analysis of variance. The ACA assay data was analyzed using repeated measures analysis of variance, described in section 4.6.

4. RESULTS/OBSERVATIONS

4.1 Cottonseed Meal Analyses

Proximate analysis and free gossypol concentrations were determined by Ralston Analytical Laboratories (St. Louis, MO) for each of the whole, ground cottonseed samples. Cottonseeds were ground with dry ice pellets (CO_2) at Monsanto in preparation for feed, analytical work and for the active cutaneous anaphylaxis assay. Samples of the ground meal were transferred by courier to Ralston Analytical Labs for analysis. Data from the individual analyses (Appendix 7.2) are summarized in Table II.

Table II. Summary data of Ralston Analytical proximate analysis of cottonseed meal samples, reported as percents of meal.

Cottonseed meal analysis	MECH-1 non-Bt	MECH-1 Bt	MECH-3 non-Bt	MECH-12 non-Bt	MECH-15 non-Bt	MECH-915 non-Bt
Moisture %	9.74	9.75	9.26	9.16	9.41	8.93
Protein % [1]	22.0	22.2	24.3	24.9	22.6	22.7
Ammonia %	0.15	0.17	0.15	0.15	0.15	0.17
Fat % (ether extractable)	14.8	14.2	16.4	17.2	15.7	16.7
Crude Fiber %	20.7	20.1	18.8	18.2	19.4	18.2
Ash %	3.62	4.16	3.68	3.80	3.86	3.65
Gossypol % [2]	0.46	0.57	n.d.	n.d.	n.d.	n.d.

[1] Protein measured by the Kjeldahl method; [2] free gossypol; n.d., not done

4.2 Transgenic Protein Expression in Seeds.

Seed samples from the six cottonseed hybrids provided by MAHYCO were ground and tested using a Cry1Ac specific solid phased immunoassay. The ground seed samples were extracted in neutral buffer and applied to the test system. Positive controls were positive for all samples. The MECH-1 Bt seed sample was the only sample with results indicating the presence of the Cry1Ac protein.

4.3 SDS-PAGE Patterns

Protein banding patterns visible after Colloidal blue staining the electrophoresis gel of the cottonseed extracts are similar (Figure 1). All identifiable bands were visible in all six cottonseed extracts, though there were minor differences in the apparent staining density for some of the bands. This qualitative test of the hybrid extracts indicates that the protein compliment from the cottonseed from the specific hybrids are similar.

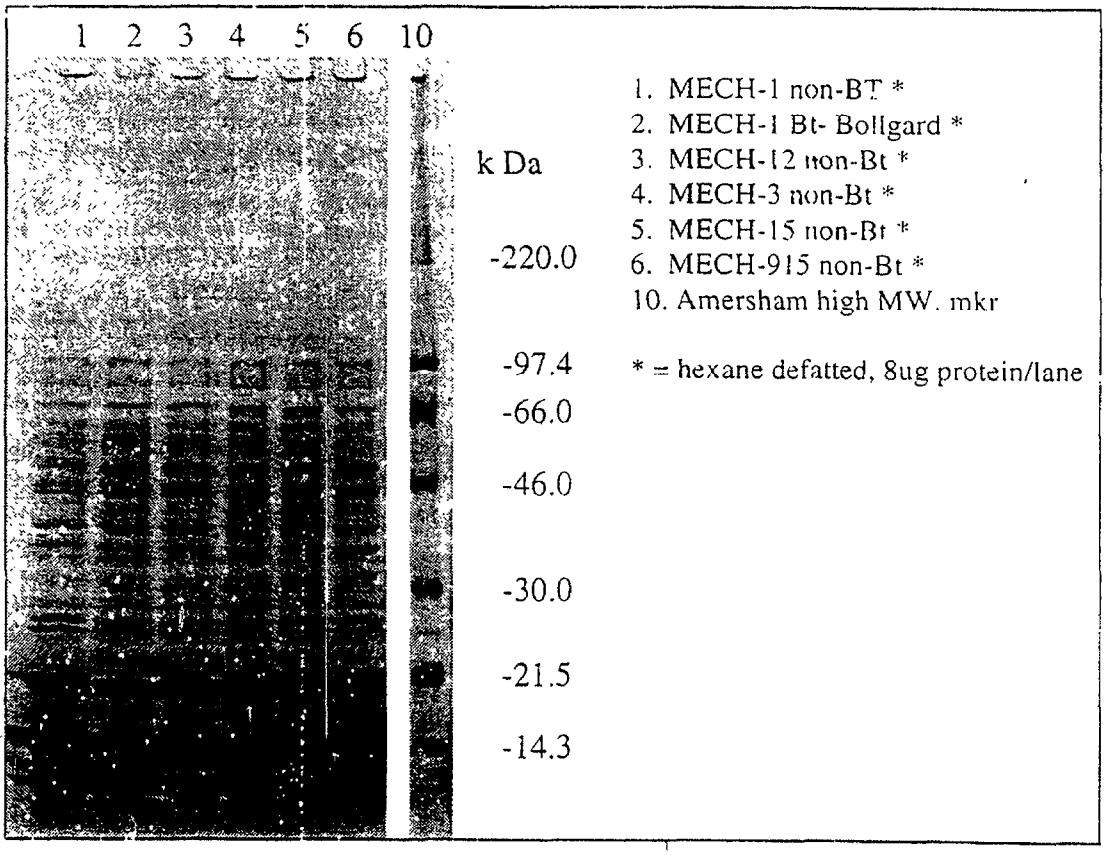


Figure 1. Colloidal blue stained SDS-PAGE gel (4-20% acrylamide) in tris glycine, of hexane defatted cottonseed extracts. The indicated sizes correspond to the marker protein bands.

4.4 Feed Manufacture

Purina TestDiet manufactured the rat feeding pellets based on the Purina 5001 rodent diet. A control diet was manufactured using the same components as the test article diets, except that no cottonseed meal was included. Portions of components of this proprietary feed were replaced with whole, raw, cottonseed meal such that either 5% or 10% of the total dietary protein was from the non-transgenic MECH-1 cottonseed meal. Other components were adjusted to provide approximately equal levels of lipid, carbohydrate and fiber as well as protein (Table III). The exact formulation was determined by the Purina TestDiet Nutritionist, based on the proximate data for the cottonseed meal provided by the Ralston Analytical Labs assay. Historical data of composition of the individual ingredients were used to formulate the diets. Contents included corn, oats, alfalfa, sugar beet pulp, wheat germ, wheat middlings, soybean meal, fish meal, meat meal, brewer's yeast, micro-nutrients, calcium carbonate, sodium chloride, whey, DL-methionine, tallow, molasses, choline chloride and water. Calculated nutritional component concentrations for the three diets are included (Appendix 7.3).

Table III. Dietary composition, percent of total mass, calculated values estimated from proximate analysis of cottonseed meal and historical data of other components.

Diets	Protein[1]	Fat (ether soluble)[1]	Fiber (crude)[1]	Starch[1]	Ash[1]
Control	22.0	4.5	6.8	27.2	6.5
5% CSM[2]	22.0	4.5	6.3	26.4	6.5
10% CSM[2]	22.0	4.5	5.7	25.9	6.5

[1] percent by weight, [2] Cottonseed meal, whole, raw.

4.5 Animal Growth Rates

All animals were marked with uniquely numbered metal ear tags prior to the start of the study, to maintain individual identities throughout the study. Each animal was weighed approximately weekly (Appendix 7.4). Periodic group weight means and standard deviations are summarized in Figure 2. Cumulative Mean percent weight gains over the 62 day feeding period (Figure 3) were compared statistically using a one-way analysis of variance. There were no significant differences among weight gains between diet groups, establishing that cottonseed diets had no adverse impact on growth rates.

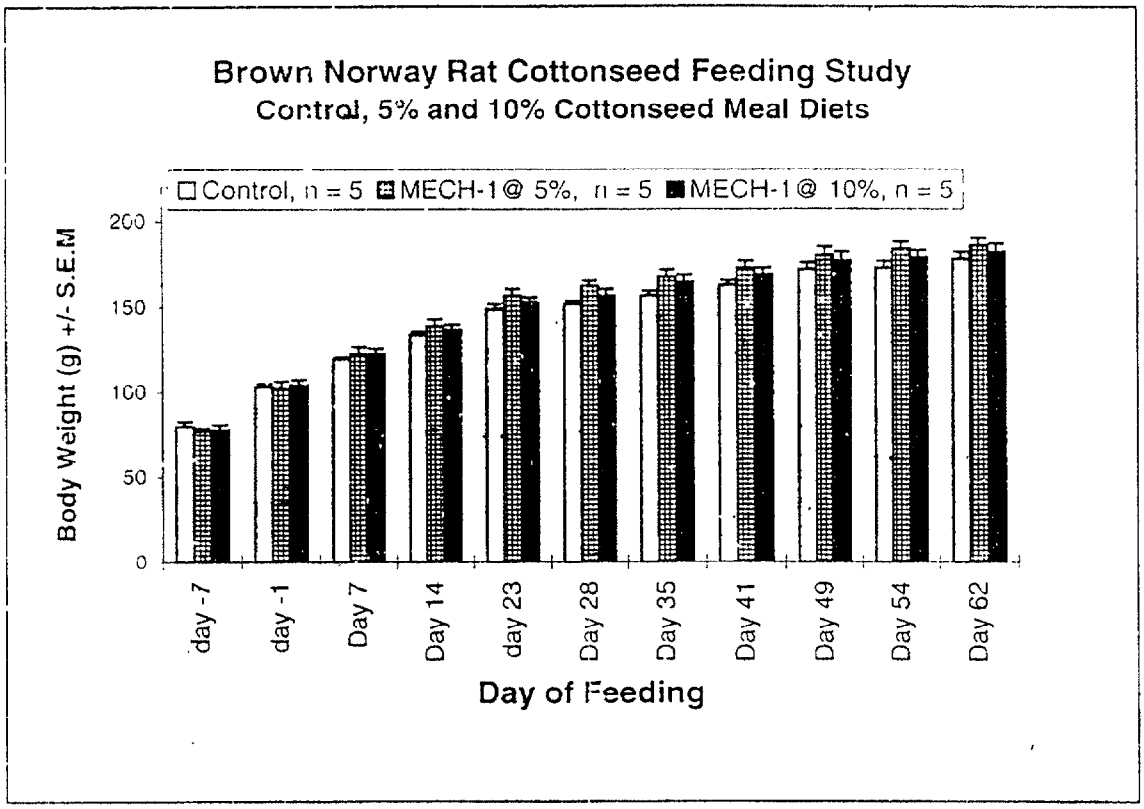


Figure 2. Average total body weights for each treatment group of Brown Norway rats, at each weight interval. Control diet was fed to the animals during the seven day acclimation period. At day 0, the animals were provided with the indicated diets, *ad libitum*, for the duration of the study.

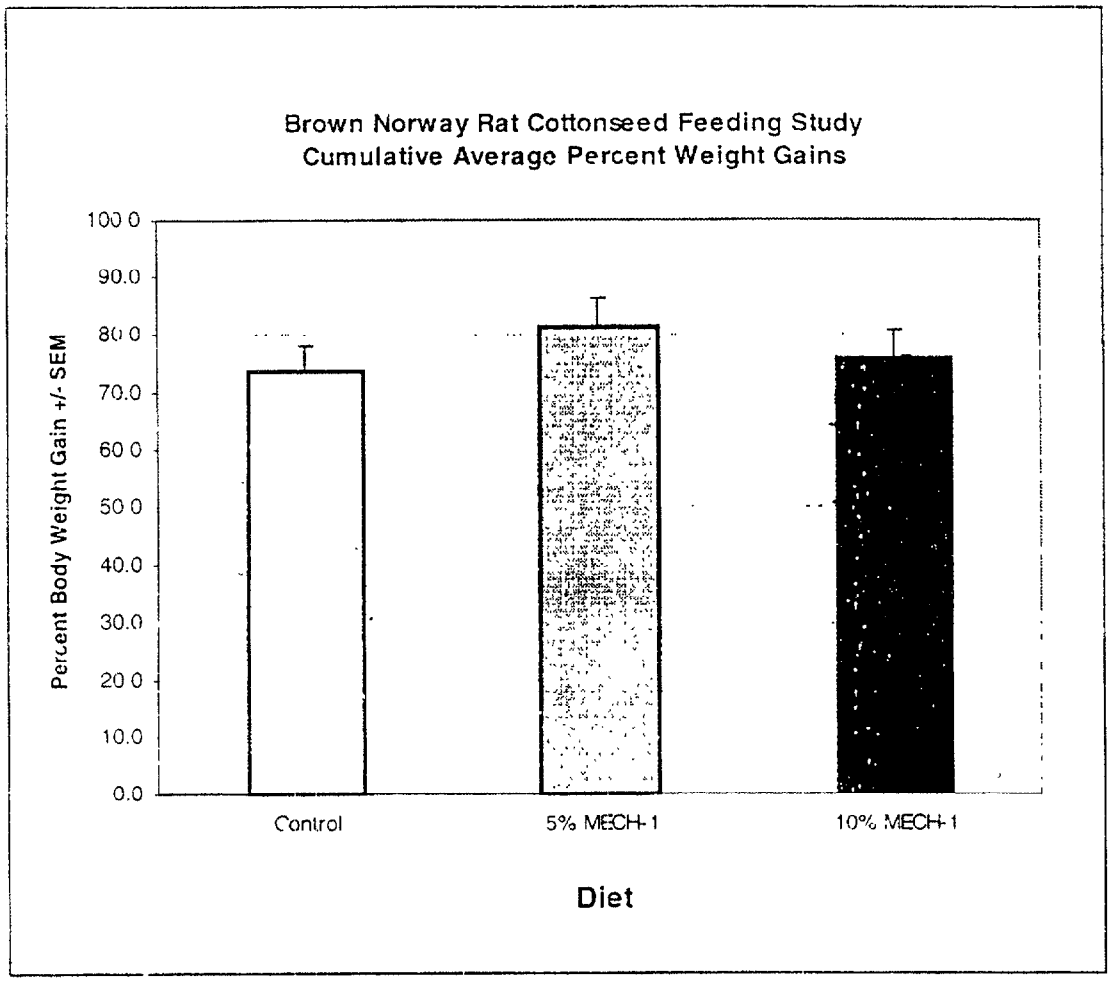


Figure 3. Cumulative averages of percent body weight gains in the three treatment groups of the Brown Norway rats, over the complete 62 day feeding period. These group differences are not statistically significant (p=0.05).

4.6 ACA Results

a. Photographs of the inner side of the animal skins (Appendix 7.5) record the dermal staining at the ACA challenge sites on each animal. There were evident animal to animal differences that appear to be random, that is, not associated with any diet, sequence of injection, or any other obvious experimental variable. However, the experiment was designed to test all four cottonseed protein extracts in each animal precisely to avoid the confounding factor of differences in individual animal responses. As expected, the response to challenges with all extracts were clearly dose dependent. The important observation is that there were no clear visual differences between the skin reactions of the same dose of each of the four

cotton extracts, compared on the same animal. This observation suggests that there are no differences between the immune or inflammatory characteristics of the four cottonseed extracts tested in this assay.

b. ACA spot diameter measurements were recorded immediately after euthanasia (Appendix 7.6). Comparison of mean values from specific diets, and at each dose confirms the visual observations, that treatment averages follow a dose-dependent response and that there were no obvious, marked differences between the four cottonseed challenge extracts (Figure 4). As indicated by the data, rats that had not been sensitized with cottonseed (control fed group) reacted to the dermal challenge doses with only slightly smaller average reactions than those from the cottonseed (MECH-1) fed rats at the same challenge doses. The response in the control diet fed animals indicates that there are inflammatory mediators in the cottonseed extracts used in the challenge that induce vascular leakage. The observed reactions involve both immune responses and inflammation.

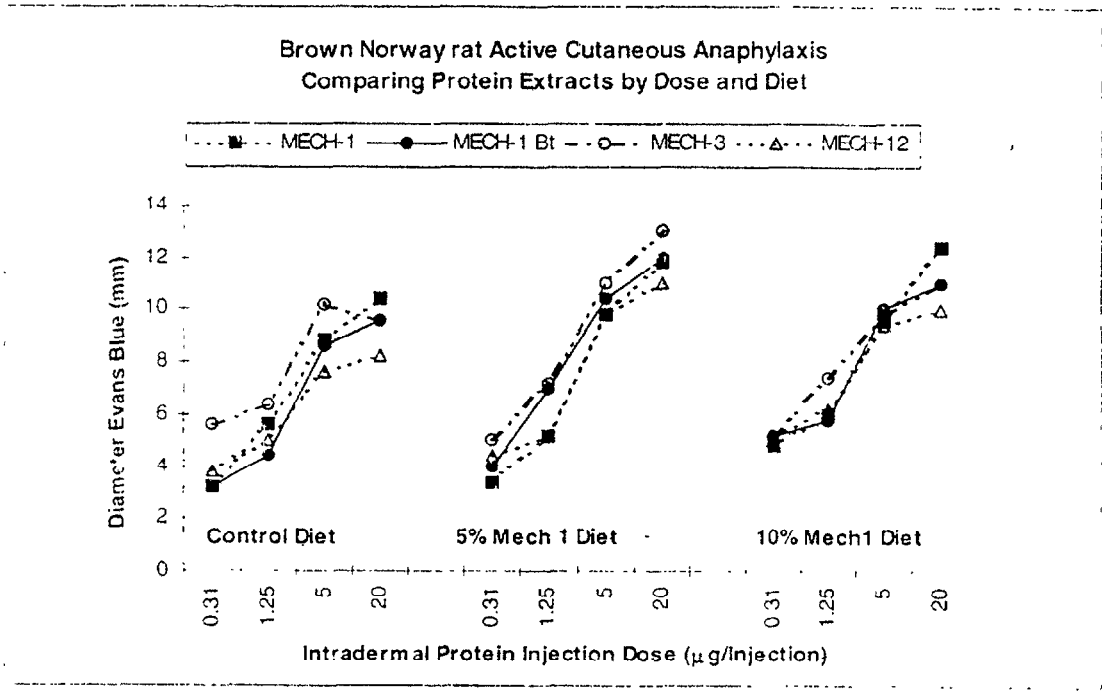


Figure 4. Average ACA spot diameters for each treatment and dose. Animals were anesthetized prior to injecting with 50 µl total volume of the diluted cottonseed meal extracts to provide the total doses indicated. Evans blue dye in saline was injected twenty minutes later and euthanized with carbon dioxide twenty minutes after the dye administration. Skins were inverted and spot diameters measured.

c. Statistical analysis of the ACA data (Appendix 7.6) was performed using a repeated measures analysis of variance on the diameters of the ACA spots, using the SAS® statistical analysis program (SAS Institute Inc., Cary, NC). This analysis was used to evaluate the significance of each of the individual components of variability, ie. diet (control, 5% MECH-1 or 10% MECH-1); dose of extract (0.31, 1.25, 5.0 or 20 µg/injection) and cottonseed protein extract source (MECH-1, MECH-1 Bt, MECH-3 and MECH-12).

The repeated measures model used is of the form:

$$D_{capd} = M_{cpd} + A_{ca} + E_{capd}$$

where

D_{capd} = diameter obtained for protein extract **p**, dose **d**, animal **a**, and cottonseed diet **c**.

M_{cpd} = true mean diameter for dose **d** of protein extract **p** for animals fed diet **c**.

A_{ca} = random error for animal **a** fed cottonseed diet **c**.

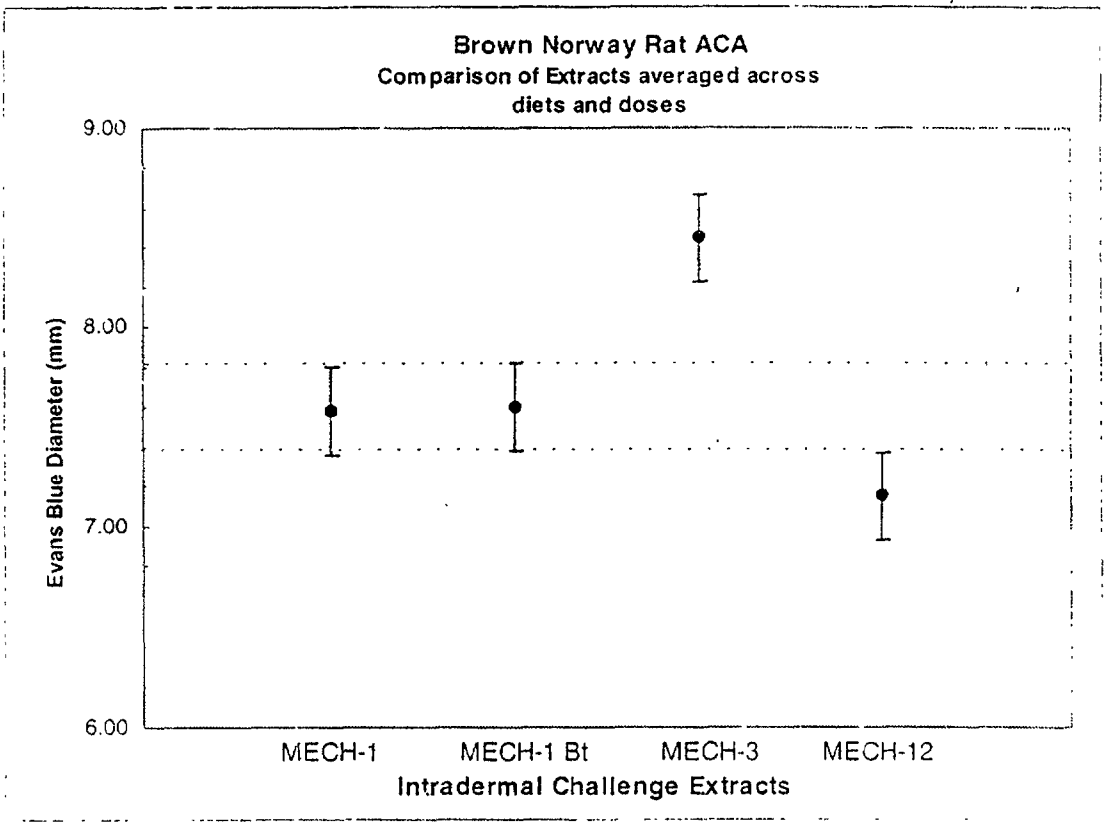
E_{capd} = random within-animal error for each observed diameter

The Mixed procedure in SAS was used to fit this mixed effect linear model to the data. The variability among the M_{cpd} was broken down into separate components representing main effects and interactions. This detailed analysis of variance is summarized in Table IV. All sources of variability are significant except the average difference between cottonseed diets and the complex 3-way interaction among diets, proteins, and doses. This means the results from all three diets may be combined to simplify the model analysis, looking for differences between cottonseed protein extracts.

Table IV. Mixed model analysis of variance summary table of comparisons of the ACA data.

Source of Variability	Degrees Of Freedom		F-value	p-value
	Numer.	Denom.		
Diets	2	12	0.61	0.5569
Proteins (challenge extract)	3	180	12.07	0.0001
Doses (challenge extracts)	3	180	369.54	0.0001
Diets × Proteins	6	180	2.17	0.0476
Diets × Doses	6	180	3.77	0.0015
Proteins × Doses	9	180	2.26	0.0202
Diets × Proteins × Doses	18	180	0.81	0.6827

The simplified analysis provides the overall critical comparison (Figure 5) between the cottonseed challenge dose extracts with data averaged across diets and across doses. As illustrated, there is no significant difference between MECH-1 and MECH-1 Bt reactivity as measured by the average diameter of Evans blue staining. The reactivity of MECH-3 was significantly greater than MECH-1 or MECH-1 Bt. The reactivity of MECH-12 was significantly less than MECH-1 Bt. The obvious conclusion is that the reactivity of MECH-1 Bt clearly falls within the range expected for proteins from non-transgenic commercial cotton hybrids and that the reactivity of MECH-1 Bt has not changed relative to MECH-1. Based on the small magnitude of the differences, it is unlikely that any of the differences are biologically important



Figures 5. Mean diameters for cottonseed challenge extracts, averaged across doses and diets. Error bars are ± one-half the 5% least significant difference between means. Therefore, any two means whose error bars do not overlap are significantly different.

Detailed statistical analysis of the individual challenge dose and challenge extract effects confirms the findings from the overall analysis. Table V

compares means obtained for each combination of diet, protein and dose. There is a clear dose response seen for all the protein extracts. A least significant difference based on a mixed model t-statistic was used to compare extract means. In only a single case (5% diet and 1.25 µg/injection) did the MECH-1 Bt differ significantly from MECH-1. No such effect was seen at any other dose or diets. Overall, the differences in mean values appear to be small and random. The possible exception is the response to MECH-3 which, in general, was found to be more reactive than the other cottonseed protein extracts.

Table V. Mean ACA diameters comparing responses to non-transgenic hybrid cottonseed extracts to those against the transgenic Bt extract, by dose and by diet. Diameter measurements are in mm. Mean values that differ by more than 1.5 mm within any specific block are significantly different ($p<0.05$).

		Control Diet		5% MECH-1 Diet		10% MECH-1 Diet	
Challenge dose	Hybrid extract	Extract Non-Bt	Bt	Extract Non-Bt	Bt	Extract Non-Bt	Bt
0.31 ug	MECH-1	3.2	3.2	3.4	4.0	4.8	5.2
	MECH-3	5.6		5.0		5.2	
	MECH-12	3.8		4.4		5.2	
1.25 µg	MECH-1	5.6	4.4	5.2	7.0	6.0	5.8
	MECH-3	6.4		7.2		7.4	
	MECH-12	5.0		5.2		6.2	
5.0 µg	MECH-1	8.8	8.6	9.8	10.4	9.6	10.0
	MECH-3	10.2		11.0		9.8	
	MECH-12	7.6		9.8		9.4	
20.0 µg	MECH-1	10.4	9.6	11.8	12.0	12.8	11.0
	MECH-3	9.6		13.0		11.0	
	MECH-12	8.2		11.0		10.0	

5. CONCLUSIONS

The overall reaginic (immune) response directed against cottonseed proteins in cottonseed fed rats was small relative to the inflammatory response as indicated by the similar level of skin reactivity in control fed rats and cottonseed fed rats. Statistical analysis and direct observation of the ACA skin reactions in Brown Norway rats show that the combined reaginic activity (allergenicity) and inflammatory characteristics of transgenic Bollgard cottonseed (MECH-1 Bt) are clearly within the range of activities observed for the group of three non-transgenic cotton hybrids. In the overall analysis, non-transgenic MECH-3 was statistically more active than transgenic MECH-1 Bt. Non-transgenic MECH-12 was statistically less active than MECH-1 Bt. It is unlikely, given the small magnitude, that these differences are biologically important. The activity of transgenic MECH-1 Bt and non-transgenic MECH-1 were not statistically different and should be considered substantially equivalent regarding allergenic activity.

The results of this study combined with the fact that: 1) there is little if any consumption of cottonseed proteins by humans; 2) that the Cry1Ac and NPTII proteins have never been implicated as allergens and do not share any significant sequence homology with known allergens; 3) these two proteins are quickly degraded in simulated gastric fluid; and 4) both proteins are expressed at very low levels in cottonseed leads to the conclusion that the allergenic risk from Bollgard cotton is no different than for commercial cotton varieties currently in use.

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APPENDICES: Assessment of the allergenicity of Bollgard cottonseed proteins relative to conventional cottonseed proteins.

Appendix 7.1 Study Protocol

Study #: 98-01-36-01

Study Title: Assessment of the Allergenicity of Bollgard Cottonseed Proteins Relative to Conventional Cottonseed Proteins.

Sponsor: Monsanto Life Sciences Company
Biotechnology Regulatory Sciences
700 Chesterfield Parkway North
St. Louis, MO 63198

Primary Testing Facility: Monsanto Life Sciences Company
700 Chesterfield Parkway North
St. Louis, MO 63198

Study Director: Richard E. Goodman, Ph.D.

Purpose.
The purpose of this study is to assess the relative allergenicity of Bollgard cottonseed proteins compared to conventional cottonseed proteins. The comparison will determine if the allergenicity of the protein components of conventional cottonseed have been altered by the introduction of the insect resistant trait into Bollgard cotton.

Experimental start date:
Proposed experimental start date: March 23, 1998

Experimental termination date:
Proposed experimental termination date: May 30, 1998

Experimental design:
Animal Model. One group of ten Brown Norway rats will be sensitized to cottonseed proteins by incorporating ground cottonseed meal from an isogenic line (of the test Bollgard line) into the feeding pellets such that 10% of the total protein intake is from cottonseed. A second group will be sensitized to the same material but with an incorporation of 5% of the total protein from cottonseed in the feeding pellets. As a negative control, a second group of 10 rats will be fed a nutrient matched, non-cottonseed diet. Animals will be fed the diets for sixty days.

Sera from the sensitized animals will be used in Western blot assays to compare IgE binding to proteins of Bollgard cottonseed, isogenic cottonseed and four other agronomic cottonseed lines. Sera from rats fed the control, non-cottonseed diet will be used as a negative control.