

Original Research Article

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Study on Skin Sensitization Potential of an Anti - tumor necrosis factor - alpha receptor fusion protein in Guinea pigs

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ABSTRACT

Etanercept is an anti-TNF α biopharmaceutical used in the treatment of several disorders of inflammation as well as autoimmune conditions. This present study was aimed to identify the skin sensitization potential of the etanercept, in guinea pigs by following Guinea Pig Maximization Test (GPMT) of Magnusson and Kligman method. The animals were divided into two groups; negative control comprising six males and 6 females and treatment groups having ten males and ten females. Through a range finding study, the dose for main study was selected as 0.5% (low concentration) and 1.0% (high concentration) of etanercept for topical induction and challenge doses. The application site was examined at 48 and 72 hours post-dosing for the presence of allergic reactions. Apart from this, animals were observed for body weight and exhibition of clinical signs of toxicity daily throughout the experimental period. At both time points of observation, no skin reaction was observed in the experimental group after the challenge. The body weights of the treated animals were found to be comparable with the control animals. Based on the results of the study, the etanercept was found to be safe and well tolerated and was concluded to be a non- sensitizer to the skin of the guinea pigs.

Keywords

Anti-TNF α
biopharmaceuticals,
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Introduction

TNF- α is identified as a key player of the vast majority of the inflammation, which are elicited in response to a wide variety of

noxious stimuli like infection, tumor malignancies etc. Although the function of TNF- α is more beneficial in acute cases, the over expression and the prolonged persistence of the circulating TNF- α leads to continuous

activation of various normal cells resulting in induction of highly detrimental chronic diseases like rheumatoid arthritis (Weinblatt *et al.*, 1999). Chronic cardiac diseases (Tziakas *et al.*, 2004), etc. Several biopharmaceutical cytokine blockers against TNF family members and TNF receptors have been developed. Some of the important TNF antagonists, which have been recommended by the FDA are infliximab, etanercept, adalimumab, certolizumabpegol and golimumab (FDA, 2011).

Etanercept is a biopharmaceutical used in the treatment of several disorders of inflammation as well as autoimmunity through the inhibition of TNF- α . Disorders of autoimmunity include conditions that arise as a result of immunological response against one's own antigens. Etanercept causes the inhibition of TNF α and thereby has the potential to treat such disorders (Feldmann and Maini, 2003). Skin sensitization is an immunologically mediated cutaneous reaction to an allergenic test substance whereby a heightened responsiveness is induced (Kimber *et al.*, 2001). Generally, skin sensitization tests are performed for the compounds that are topically applied or that have the chance of unintentional exposure to skin surface. However, it is also warranted to the injectable substance especially for those that are injected subcutaneously as these substances stay at subcutaneous area for a sufficient period of time that facilitates the elicitation of sensitization reaction. Further, human sensitization tests are time consuming and very expensive because a large number of volunteers (around 150-200) is required for each test and moreover, the participated volunteers form an inhomogeneous test group which may alter the test data (Basketter *et al.*, 2004).

In the assessment and evaluation of the toxic characteristics of a test substance,

determination of its potential to provoke skin sensitization reactions in a homogeneous group of animal models becomes a mandate. The guinea pig maximization test (GPMT) is a more sensitive assay and have been accepted for hazard identification of skin sensitizing substances (Magnusson and Kligman, 1969 and Basketter and Scholes, 1992). The maximization test is an 'adjuvant' type test in which the sensitization is potentiated by the intra-dermal injection of Freund's Complete Adjuvant. Since the recombinant etanercept is clinically intended through subcutaneous route in humans, the guinea pig maximization test was performed to assess the skin sensitization potential of etanercept in the guinea pig.

Materials and Methods

Animals & Husbandry

The guinea pig (*Cavia porcellus*; Dunkin Hartley) was selected as the test system as the skin of guinea pig is more similar to human skin and is historically shown to be suitable model for skin sensitization potential testing. A total of twelve guinea pigs (six males and six females) were used for range finding study. For main study, thirty two animals were divided into control (six males and six females) and treatment (ten males and ten females) groups. Prior to the start of the experiment, the animals were housed in their respective cages in experimental room for 5 days so as to acclimatize with the laboratory conditions. During the whole experimentation, the experimental room temperature was maintained between 17 and 20 °C and the relative humidity were between 45 and 60%. Temperature and relative humidity were monitored once daily. The experimental room was provided with a 12 h light and 12 h dark photoperiod cycle controlled by an automatic timer. Test room light intensity and the noise level were

maintained within 100 lux and 50db, respectively. Gamma irradiated rodent pellet feed supplied by Laboratory Animal Feed, Pune, India and clean reverse osmosis purified drinking water was provided to the animals at *ad libitum*. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) and Institutional Bio-safety Committee (IBSC) and the study was conducted at International Institute of Biotechnology and Toxicology, Padappai.

Testing Method

Guinea Pig Maximization Test (GPMT) of Magnusson and Kligman (1969) method was followed.

Preparation of the Test Substance

For intradermal injection, the test substance was dissolved, in a formulation buffer. For the intradermal induction phase of the main study, incorporation of the test substance in a 1:1 mixture (v/v) of FCA (Freund's Complete Adjuvant) and formulation buffer was used. For topical applications, the test substance was applied as such.

Range Finding Study

Intradermal Induction

The fur over the dorsum of the animals was neatly clipped without causing abrasion. The etanercept at 0.5% solution of 0.1ml was intradermally injected over the clipped flank region of four guinea pigs (2 males + 2 females). The injection site was examined for pathological lesions at approximately 24, 48 and 72 h and five days after intradermal injection. As there was no dermal reaction observed in animals injected with 0.5% solution of 0.1 ml of test substance, the same concentration (i.e. 0.5% solution of 0.1ml)

was selected for intradermal induction phase of the main study.

The etanerceptas 0.5 % solution of 0.2 ml (1 male + 1 female) and as 1.0 % solution of 0.2 ml (1 male + 1 female) was prepared and applied over the clipped area of the animals as occlusive patch. After 48 h exposure period, the degree of erythema and oedema was evaluated using the grading scale specified in Table 1. As none of the animal exhibited any skin reaction at 1, 24 and 48 h after removal of the patch, the highest concentration of 1.0 % solution of 0.2 ml was selected for the topical induction and challenge phases of the main study.

Main study

The procedure involved treatment in two phases i.e. induction and challenge phase.

Induction Phase of Control Group

Intradermal Injection

Injections were given on day 0 using the procedures described for test group animals except that the pairs of injections were:

Injection 1: 1:1 mixture (v/v) FCA/distilled water

Injection 2: Formulation buffer alone

Injection 3: 50% v/v formulation buffer in a 1:1 mixture (v/v) FCA/distilled water.

Topical Application (Day 7)

Control animals were treated identically to the test group animals with formulation buffer. Approximately 48 h after application, the occlusive dressings were removed from each control group animal. The skin reactions were evaluated approximately 1 and 24 h after dressing removal, as described for the treatment group animals.

Induction Phase of Treatment Group

Intradermal Injections (Day 0)

Prior to the administration of etanercept, a small area over the shoulder region of the animals was clipped without causing any abrasion. Three pairs of intradermal injections of 0.1 ml volume were given in the shoulder region. Injections of 0.1 ml volume of each pair lies on each side of the midline.

Injection 1: 1:1 mixture (v/v) FCA/distilled water

Injection 2: 0.5% solution of the etanercept in formulation buffer

Injection 3: 1.0% solution of the etanercept in formulation buffer formulated in a 1:1 mixture (v/v) FCA/distilled water.

Injections 1 and 2 were given close to each other nearest to the head, while injection 3 was given at the caudal part of the test area. Approximately 24 and 48 h after intradermal injection, the animals were observed for pathological alterations, if any, at the test substance injection sites according to the scale given in the Table 1.

Topical Induction (Day 6 and 7)

The induction site was clipped free of hair. Since, the test substance was found to be not a skin irritant, 0.5 ml of 10% sodium lauryl sulphate in vaseline was applied to the test area of control and treated groups, in order to create a local irritation on day 6. On the next day (day 7), a filter paper (approximate size 2 x 4 cm) was loaded with test substance (1.0% solution). The loaded patch was applied to the clipped induction site and held in place with adhesive tape. The patch was covered with occlusive dressings and secured with adhesive band in a double layer around the shoulder region. Approximately 48 h after application, the occlusive dressings were removed from

each test group animal and the skin reactions were evaluated approximately 1 and 24 h after dressing removal.

Topical Challenge Application in control and treatment group

Topical Application (Day 21)

Shortly before treatment, an area approximately 5 cm x 7 cm on the shoulder region was clipped. Care was taken to avoid abrasion of the skin. A filter paper patch (Whatman No.4; approximate size 2cm x 2cm) was loaded with the 0.5% of 0.2 ml etanercept solution at the maximum non-irritant concentration and was applied to the clipped right flank of G2 treated group and to the left flank of G1 control group animals. The patch was held in place with adhesive tape. All patches were covered with an adhesive bandage. Approximately 24 h after application, all occlusive dressings were removed. The challenge sites were gently swabbed using cotton wool soaked in distilled water to remove excess test substance. Care was taken not to alter the existing skin response. The position of the challenge sites was identified using a black indelible marker pen.

Evaluation of Challenge Reactions

Approximately 21 h after removal of patches, the challenge test sites were clipped free of hair and approximately 48 and 72 h after application of challenge patch, the degree of erythema and edema were evaluated according to the Table 1.

Observations

Body weight

Body weight of individual animal was recorded prior to the induction exposure and

at the end of the experimental period.

Toxicity Signs

Animals were observed for clinical signs of toxicity of convulsion, coma, death, eschar formation, exophthalmos, hyperactivity, piloerection, polyuria, prostration, wound formation, alopecia, diarrhea, lacrimation, respiratory distress, catalepsy, chromodacryorrhea, dullness, edema, nasal irritation, nostril discharge, rigidity, salivation, change of body coat color, erythema, paralysis and tremor daily throughout the experimental period.

Euthanasia

On termination, the experiment animals were euthanized by using CO₂.

Results and Discussion

Body weight

Normal body weight was observed in the test substance treated guinea pigs and control group animals (Table 2).

Toxicity Signs

No clinical signs of toxicity were observed. All animals were apparently healthy throughout the experiment.

Skin Reactions

None of the animals in treatment and control groups presented any skin reaction at 48 and 72 h after application of the challenge patch. Since none of the animals in treatment and control groups presented any erythematous responses, a grade of '0' was given to all the animals at both the time points of observation after the challenge patch application (Table 3).

Non-communicable chronic inflammatory diseases like IBD, neoplasm, diabetic complications and degenerative disorders of lungs, heart and brain have been observed to have increasing incidences and are attributed as the major cause of death, globally. Unhealthy food patterns, smoking, lack of physical activity, stress, radiation exposure and environmental pollutants are regarded to be the frequent causes of chronic disorders. Majority of such risk factors have been observed to be closely related to chronic inflammation that can result in several chronic disorders (Prasad and Aggarwal, 2014). From the study it was concluded that etanercept was not a skin sensitizer in guinea pigs. As per the Centers for Disease Control and Prevention, USA, such disorders result in around for 63% of total mortality globally and around two-thirds of mortality (17 lakhs every year) in the United States. Traditionally, the treatment of chronic inflammatory disease has been to target the inflammatory response. The various substances that can achieve the anti-inflammatory effect have in common that they inhibit the activity of the immune system, although for many of them, the exact mechanism remains elusive. The main drawback with immunosuppressive treatment is the increased risk of infection arising from the manipulation of the immune system. It is believed that corticosteroids act by entering into immune cells to inhibit genes that code for pro-inflammatory cytokines, among them mainly, TNF (Spies *et al.*, 2010).

Advances in our understanding of the immune system, as well as the advent of the era of biotechnology, have triggered great interest in the development of new therapies for inflammatory disorders. Our better understanding of these disorders has also shifted treatment strategy from a more conservative approach to a much more aggressive one. Although conventional

treatment modalities remain the mainstay and are sufficient and appropriate in many and may be the majority of patients, we have clearly entered the “biologic” treatment era. Compared to conventional treatment, these agents target the immune system more selectively and therefore have fewer non-specific side effects, although many cytokines are certainly pleiotropic.

The introduction of anti-TNF treatment has been a significant addition to the available treatment regimens of chronic inflammatory disease and has also added valuable insight in disease pathogenesis (Sfikakis, 2010). These drugs are aimed to particularly target TNF that plays the central role in chronic inflammation and currently, 5 drugs namely, infliximab, adalimumab, etanercept, certolizumab-pegol and golimumab are used. Despite their common target, their effects also differ. Etanercept is effective for rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis and psoriasis, but not for Crohn’s disease or ulcerative colitis (Sfikakis, 2010 and Sandborn *et al.*, 2001). Common for all anti-TNF substances is that their indication depends on disease severity, and they are generally indicated in moderate to severe disease.

Etanercept is a biopharmaceutical used in the treatment of several disorders of inflammation as well as autoimmunity through the inhibition of TNF- α . Disorders of autoimmunity include conditions that arise as

a result of immunological response against one’s own antigens. TNF α is a cytokine secreted by the T cells, B cells as well as the mononuclear phagocytes. TNF α promotes inflammation further through the recruitment of leucocytes to the zone of inflammation and through the initiation and activation of other molecular pathways that amplify inflammatory response. Etanercept causes the inhibition of TNF α and thereby has the potential to treat such disorders (Feldmann and Maini, 2003). It is a fusion protein synthesized through recombinant DNA technology which binds the tumor necrosis factor receptors (TNFRs) to the constant domain of the immunoglobulin, Ig G1.

Preclinical toxicology evaluation of biopharmaceuticals, however, present significant challenges to human risk assessment owing to their innovative and complex nature (Brennan *et al.*, 2015). The major objectives of preclinical assessment of biopharmaceuticals are (1) to identify target organs showing toxic effects and to ascertain whether the toxic effects are reversible after the cessation of drug administration, (2) to identify a safe starting dose in case of human Phase I clinical trials and for further dose escalation, (3) provision of data to assist the monitoring of safety parameters in human trials and (4) provision of safety data to strengthen the claims on the product label (Cavagnaro, 2008).

Table.1 Skin reaction grading scale*

| Observations | Grades |
|---------------------------------|--------|
| No visible change | 0 |
| Discrete or patchy erythema | 1 |
| Moderate and confluent erythema | 2 |
| Intense erythema and swelling | 3 |

*Magnusson and Kligman grading scale for the evaluation of challenge patch test reactions

Table.2 Individual Animal Body Weight Data

| Group | Animal No. | Sex | Body weight (g) | |
|--|------------|--------|-----------------|--------|
| | | | Day 0 | Day 24 |
| G1 - Control Group | SS 01 | Male | 256 | 279 |
| | SS 02 | | 272 | 295 |
| | SS 03 | | 273 | 298 |
| | SS 04 | | 262 | 286 |
| | SS 05 | | 257 | 280 |
| | SS 06 | | 258 | 281 |
| G1 - Control Group | SS 07 | Female | 269 | 294 |
| | SS 08 | | 255 | 276 |
| | SS 09 | | 265 | 288 |
| | SS 10 | | 256 | 286 |
| | SS 11 | | 256 | 284 |
| | SS 12 | | 256 | 288 |
| G2 – Treatment Group (Etanercept @ 0.5% and 1.0% concentration) | SS 13 | Male | 258 | 279 |
| | SS 14 | | 244 | 268 |
| | SS 15 | | 246 | 268 |
| | SS 16 | | 266 | 288 |
| | SS 17 | | 268 | 285 |
| | SS 18 | | 246 | 269 |
| | SS 19 | | 255 | 285 |
| | SS 20 | | 253 | 284 |
| | SS 21 | | 266 | 288 |
| | SS 22 | | 255 | 278 |
| G2 – Treatment Group (Etanercept @ 0.5% and 1.0% concentration) | SS 23 | Female | 264 | 284 |
| | SS 24 | | 266 | 288 |
| | SS 25 | | 248 | 270 |
| | SS 26 | | 259 | 275 |
| | SS 27 | | 265 | 290 |
| | SS 28 | | 266 | 291 |
| | SS 29 | | 259 | 275 |
| | SS 30 | | 258 | 276 |
| | SS 31 | | 264 | 284 |
| | SS 32 | | 259 | 279 |

Table.3 Evaluation Of Challenge Patch Test Reactions

| Group | Animal No. | Sex | Hours (after application of challenge patch) | |
|--|------------|--------|--|----|
| | | | 48 | 72 |
| G1 - Control Group | SS 01 | Male | 0 | 0 |
| | SS 02 | | 0 | 0 |
| | SS 03 | | 0 | 0 |
| | SS 04 | | 0 | 0 |
| | SS 05 | | 0 | 0 |
| | SS 06 | | 0 | 0 |
| G1 - Control Group | SS 07 | Female | 0 | 0 |
| | SS 08 | | 0 | 0 |
| | SS 09 | | 0 | 0 |
| | SS 10 | | 0 | 0 |
| | SS 11 | | 0 | 0 |
| | SS 12 | | 0 | 0 |
| G2 – Treatment Group (Etanercept @ 0.5% and 1.0% concentration) | SS 13 | Male | 0 | 0 |
| | SS 14 | | 0 | 0 |
| | SS 15 | | 0 | 0 |
| | SS 16 | | 0 | 0 |
| | SS 17 | | 0 | 0 |
| | SS 18 | | 0 | 0 |
| | SS 19 | | 0 | 0 |
| | SS 20 | | 0 | 0 |
| | SS 21 | | 0 | 0 |
| | SS 22 | | 0 | 0 |
| G2 – Treatment Group (Etanercept @ 0.5% and 1.0% concentration) | SS 23 | Female | 0 | 0 |
| | SS 24 | | 0 | 0 |
| | SS 25 | | 0 | 0 |
| | SS 26 | | 0 | 0 |
| | SS 27 | | 0 | 0 |
| | SS 28 | | 0 | 0 |
| | SS 29 | | 0 | 0 |
| | SS 30 | | 0 | 0 |
| | SS 31 | | 0 | 0 |
| | SS 32 | | 0 | 0 |

*Number indicates the skin reaction scoring as mentioned in Table 1.

The preclinical toxicity evaluations have to be conducted using young healthy rodents / non-

rodents complying with the regulatory guidelines of Good Laboratory Practice

(GLP) standards (OECD, 1998; FDA, 2010). These studies do not characterize pharmacology, which is generally analysed using specifically designed efficacy studies before the initiation of GLP toxicity evaluations. These studies are, in general, performed in 2 species, a rodent species and a non-rodent species (ICH, 2009).

The objective of such investigations is to assess the possible target organs of toxicity as well as to ascertain the dose wherein adverse effects are absent. The ratio of the maximum tolerated dose to that of the no observed adverse effect level gives us a measure of safety margin of the test item. Toxicity is classified as either 'on-target', i.e., direct pharmacological effects of the drug, or "off-target", i.e., indirect non-pharmacological effects (Guengerich, 2011). Skin sensitization test describes an immunological process whereby excessive response to any allergen is induced. To determine the skin sensitization potential of the etanercept, guinea pigs were selected as the model. All treated animals showed normal body weight gain and no adverse skin reaction was observed in the experimental group after the challenge at both the time points of observation. Skin sensitization potential study of etanercept in guinea pigs revealed no clinical signs of toxicity. No skin reaction was observed in the experimental group after the challenge at both the time points of observation. Normal body weight was observed among all the animals of the treated groups. Etanercept was found to be safe and well tolerated in this study and was concluded to be a non-sensitizer to the skin of the guinea pigs.

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