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Investigation of the sensitisation potential of various textile dyes using a biphasic mice local lymph node assay (LLNA) and an in vitro loose-fit coculture-based sensitisation assay (LCSA)

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1 Introduction

1.1 Allergy

Allergy is a disorder of the immune system. The concept ‘allergy’ was introduced in 1906 by the Viennese paediatrician Clemens Freiherr von Pirquet, when he observed hypersensitivity in his patients to normally harmless entities such as dust, pollen, or certain foods. Pirquet named this phenomenon ‘allergy’ from the Greek words *allos* meaning ‘other’ and *ergon* meaning ‘work’ (Von Pirquet, 1906). At that time, all forms of hypersensitivity were classified as allergies, and were supposed to be caused by an improper activation of the immune system. In 1963, a new classification scheme was introduced by Philip Gell and Robin Coombs that described four types of hypersensitivity reactions (Table 1.1), known as Type I to Type IV hypersensitivity (Gell and Coombs, 1963). According to this classification, the word ‘allergy’ was restricted to only type I hypersensitivity.

Type	Name	Disorders observed	Mediator
I	Allergy/ Immediate type	Atopy, Anaphylaxis, Asthma	IgE
II	Cytotoxic, Antibody-dependent	Autoimmune haemolytic anemia, Thrombocytopenia, Erythroblastosis fetalis, Myasthenia gravis	IgM or IgG, complement
III	Immune complex disease	Serum sickness, Arthus reaction, SLE	IgG, complement
IV	Delayed type hypersensitivity (DTH), cell-mediated	Contact dermatitis, Chronic transplant reaction, Multiple sclerosis, Tuberculin reaction	T-cells

Table 1.1 Gell and Coombs classification of allergy

1.1.1 Contact dermatitis

Contact dermatitis is an inflammatory reaction of the skin characterized morphologically by an eczematous reaction – erythema, vesicles, exudation, papules, squames and exsiccation being present sequentially or simultaneously (Brasch et al., 2007). It is usually induced by external non-infectious immunological, chemical or physical factors.

There are three types of contact dermatitis: irritant contact, allergic contact, and photocontact dermatitis.

1.1.1.1 Irritant contact dermatitis

Irritant contact dermatitis (ICD) is caused as a result of non-specific cellular damage to the skin, which may be either chemical or physical in origin. Allergens, although being structurally dissimilar, seem to provoke common molecular events during the induction and elicitation of allergic contact dermatitis (Enk and Katz, 1992). On the other hand, irritants initiate inflammation by various mechanisms, dependent upon the physicochemical properties of the irritant and the circumstances of exposure (Basketter et al., 1997; Wilkinson and Willis, 1998). These mechanisms may comprise disruption to the stratum corneum barrier resulting in increased water loss and/or penetration of irritant substances, disruption of cellular membranes in the epidermis leading to synthesis of proinflammatory prostaglandins etc., alteration of keratinocytes causing release of proinflammatory cytokines, cytotoxicity leading to the release of mediators/tissue destructive enzymes etc., direct effects on dermal blood vessels and cell surface adhesion molecules leading to production of an inflammatory filtrate.

Common chemical irritants include sodium dodecyl sulphate, benzalkonium chloride, croton oil, acetone, dinitrochlorobenzene, sodium hydroxide, potassium dichromate, toluene, trichloroethylene etc. (Basketter et al., 1999a).

1.1.1.2 Allergic contact dermatitis

Allergic contact dermatitis (ACD) is the manifestation of an allergic response caused by contact with a substance. ACD is accepted to be the most prevalent form of immunotoxicity found in humans (Kimber et al., 2002).

The recent understanding of events of the sensitisation process probably began to unfold after the patch test was developed by Jadassohn (Jadassohn, 1895). The next important episode involved the passive transfer of immediate-type allergy by the Prausnitz-Kustner reaction (Prausnitz and Kustner, 1921). This involved injecting blood serum from an allergic person into the skin of a normal person, making the injected site reactive to the injected

allergen. Landsteiner and Jacobs demonstrated that delayed-type reactions could be induced by intradermal injection of certain allergens (Landsteiner and Jacobs, 1936). Another important finding was that of Landsteiner and Chase that lymphocytes become sensitised during the development of ACD (Landsteiner and Chase, 1942). The development of predictive and diagnostic human and guinea pig tests for skin sensitisation drew further attention on ACD (Schwartz, 1941), regulatory and legal requirements for evaluating drug and cosmetic safety (Draize, 1959) also played significant role.

Factors which influence whether an individual develops ACD include: exposure time, method of exposure, concentration of allergen, genetic susceptibility.

1.1.1.3 Photocontact Dermatitis

Photocontact dermatitis (PCD) is triggered by an interaction between a non-harmful or less harmful substance on the skin and ultraviolet light (320-400nm UVA), thus manifesting itself only in regions where the patient has been exposed to rays (Bourke., et al 2001). It is divided into two categories, phototoxic and photoallergic. The mechanism of action varies from chemical to chemical, but is usually due to the production of a photoproduct. Phototoxic and photoallergic reactions can be diagnosed separately on the basis of pathogenesis, clinical characteristics, and histology. Examples of drugs capable of inducing a phototoxic reaction include amiodarone, retinoids, nonsteroidal anti-inflammatory agents, diuretics, and antibiotics. Substances known to cause a photoallergic response are fragrances, sunscreens, topical antimicrobials, NSAID, and psychiatric medications, such as chlorpromazine (Zhai and Maibach, 2004).

1.1.2 Pathophysiology of allergic contact dermatitis

ACD is termed a Type IV delayed hypersensitivity reaction involving a cell-mediated allergic response. Allergic contact dermatitis (ACD) is caused by small chemical molecules (haptens) that weigh less than 500 Da (Eisen et al., 1952). Haptens have the physico-chemical properties that allow them to cross the stratum corneum of the skin. They can only cause their response as part of a complete antigen, involving their association with epidermal proteins forming hapten-protein conjugates. This requires them to be protein-reactive. ACD comprises of two essential stages: an *induction phase*, which primes and sensitizes the

immune system for an allergic response, and an *elicitation phase*, in which this response is triggered (Belisto, 1989; Bergstresser, 1990).

Phase 1 – Sensitization phase (afferent phase or induction phase)

This occurs at the first contact of skin with the hapten. After penetrating the stratum corneum, haptens are processed and displayed on epidermal Langerhans cells (LCs) bearing multiple surface receptors including CD1a, major histocompatibilty complex (MHC), Fc, and complement receptors (Roitt et al., 2001). Following uptake of allergens or allergen-protein complexes through pinocytosis or receptor-mediated endocytosis, LCs up-regulate expression of surface molecules such as MHC and co-stimulatory factors including interleukin-1 and TNFα (Heufler et al., 1988; Aiba and Katz, 1990; Austyn et al., 1983), intercellular adhesion molecule-1 (ICAM-1), and secretion of immunomodulatory cytokines (Rietschel and Ray, 1988). As the LCs are transported to the lymph nodes, they become differentiated and transform into dendritic cells (DCs), which are immunostimulatory in nature. The end result of ACD is activation of a subset of T cells with unique T-cell receptors (TCRs) specific for the antigen. Both contact allergens and irritants can activate keratinocytes in this way, but only allergens are thought to activate LCs adequately, resulting in effective presentation of antigen to T cells. T cells proliferate and emigrate out of the lymph nodes to the blood where they recirculate between the lymphoid organs and the skin. These T cells divide and differentiate, clonally multiplying so that if the individual is exposed again to the allergen, these T cells will respond more quickly and aggressively (Kimber et al., 2002). The sensitisation step lasts 10 to 15 days in man, and 5 to 7 days in the mouse (Saint-Mezard et al., 2004).

Phase 2 - Elicitation phase (efferent or challenge phase)

Challenge of sensitized individuals with the same hapten leads to the appearance of ACD. Haptens diffuse in the skin and are uptaken by skin cells which express MHC I and II/haptenated peptide complexes. The elicitation response results due to inflammatory effects of cytokines, including TNFα and IL-1 (Belsito, 1989; Bergstresser, 1990), which are potent inducers of endothelial adhesion molecules including ICAM-1, selectins, integrins, leukocyte functional antigen-1 (LFA-1), and very late antigen-4 (VLA-4) (Roitt et al., 2001). Antigen exposure involves two opposing pathways. The first is mediated by effector T cells, resulting in a state of hypersensitivity exhibited as an eczematous skin reaction i.e. ACD. The other

pathway leads to production of suppressor T cells, which mediate antigen tolerance. Suppressor T cells have been shown to be CD4+ and CD25+, similar to T cells recognized to play active part in preventing auto-immunity and graft rejection. Skin reactivity against specific allergens depends on the balance between the effector and suppressor T cells. Specific T-cells are activated, which elicit the inflammatory process accountable for the cutaneous lesions. The efferent phase of ACD takes 72 hours in man, and 24 to 48 hours in the mouse. The inflammatory reaction persists only for a few days and rapidly decreases following down-regulatory mechanisms (Saint-Mezard et al., 2004).

1.1.3 Textile dyes as contact allergens

Allergies to textiles are likely under-diagnosed (Guiraud-Pons, 1999). This may be due to under reporting by the patient, the difficulty in correlating a dermatitis with textiles and the diverse clinical presentations of a textile allergy (Smith and Gawkrodger, 2002), for which sweating and friction are the activating or aggravating factors (Rietschel and Fowler, 2001). Dyes are the first, and resin finishes are the second major causes of textile dermatitis; their presence being dependent on the textile fabric composition. Chemicals such as metals, lubricants, fragrances, biocides and UV absorbers may be other potential textile allergens.

Reports of dye-related contact sensitization include exposure to dyes in clothing (Brandao et al. 1985), cosmetics (Calnan 1976), spectacle frames (Dooms-Grossens et al. 1981), felt-tip marker pens (Miller et al. 1978), shoes (Correia and Brandao 1986) and a diversity of other consumer products. Contact sensitization due to occupational exposure has been reported during the manufacturing of hand-printed fabrics and in clothing factories where workers remain in direct contact with newly dyed garments (Cronin 1980).

Disperse dyes, of which 32 have been documented (Hatch et al. 2003), are the main causative agents for textile dermatitis. They are used on synthetic fibres such as polyesters, acetates, triacetates and nylon or on a combination of fabrics (Lepoittevin 1999). Due to their lipophilic feature and molecular size these dyes have easy skin penetration, which is a requirement for inducing ACD. When used to stain polyamide or acetate, the fastness of disperse dyes to the fibre is limited. If the fastness to perspiration is insufficient, dyes with a sensitising potential bear a risk of allergic reactions including contact dermatitis, particularly when used in skin-tight garments (Specht and Platzek, 1995; Platzek, 1996). Certain disperse dyes have been recognized as triggering substances, mainly when used in skin-

tight garments made of chemical fibres (Hatch and Maibach, 1985, 1995; Hatch, 1995). This phenomenon was referred to as “stocking dermatitis” (Hausen and Schulz, 1984) or “leggins allergy” (Elsner, 1994). A few reports of dermatitis due to other dyes can be found in the literature.

1.2 In vivo models for sensitisation studies

For many decades, the guinea pig was the animal of choice for predictive studies of skin sensitisation potential. This was largely because of the use of the guinea pigs in the pioneering investigations into mechanisms of skin sensitisation to chemicals (Landsteiner and Jacobs, 1935, 1936). The guinea pig maximisation test (GPMT) and the Buehler test, are the most widely used and accepted procedures as per the regulatory guidelines (JMHW, 1993; OECD, 1993; Seabaugh, 1994; EC, 1996).

1.2.1 Guinea pig maximization test

In the guinea pig maximization test (GPMT), as described by Magnusson and Kligman (1969, 1970), the induction phase consists of two phases. It is initiated (day 0) by paired intradermal injections into the shaved shoulder region of complete Freund’s adjuvant (FCA), test chemical in vehicle, mixture of dissolved or suspended test chemical with FCA. On day 7, a topical occlusive patch is applied for 48 hour on the clipped shoulder region. Challenge is performed on day 21. On the left shaved flank of all animals, the test chemical is applied in vehicle at non-irritating concentrations using a 24 hour occlusive patch. The challenge reaction examined 24 and 48 hour after removal of the patch, scored according to standard rating scale, and the classification of allergenic potential is graded from none to extreme. Control animals are treated similar to test animals, except that during the induction phase the test chemical is not used.

GPMT is a very sensitive method for allergenicity screening of chemicals (Andersen, 1993; Anderson et al., 1985; Andersen and Maibach, 1985) with a tendency to overestimate the potency of many weak, mild and moderate human sensitisers. Due to its limits, various modifications of the GPMT have been proposed (Sato et al., 1981; Maurer and Hess, 1989).

1.2.2 Buehler test

In the standard procedure of the Buehler test (Bühler, 1985; Ritz and Bühler, 1980), the induction phase is performed by 1 or 3 weekly occlusive applications of the test chemical at slight to moderately irritating concentration. On the left clipped shoulder, occlusive patches with the test chemical at the highest possible (moderate irritating) or anticipated use concentration are fixed to the skin for 6 hours. For the challenge phase, following a rest period of 14 days after the last induction exposure, test and control animals are treated with a nonirritating concentration of the test chemical to naive clipped back skin under an occlusive patch for 6 hour. During the induction and challenge procedure the animals are kept in a specially designed restrainer, which prevents their movement and enables attachment of the occlusive patch. The observations and grading of skin reactions are done 24 and 48 hour after challenge. This assay is uneconomical, time-consuming, and the validity of results is usually limited to the used concentration.

The guinea-pig methods have drawbacks, discomfort to animals, often poor reproducibility and present difficulties of interpretation. During the last three decades, there has been a growing interest in the use of mice for experimental investigations of contact hypersensitivity. The methods used principally for assessing the sensitising potential using the mouse as a model include popliteal lymph node assay, mouse ear swelling test and local lymph node assay.

1.2.3 Popliteal lymph node assay

The popliteal lymph node assay (PLNA) is based on changes in the lymph node draining the footpad. Procedures of the PLNA involve either mice (Kammüller et al., 1989) or rats (Verdier et al., 1990). Experimental animals are subcutaneously injected with the test compound in one hind footpad; identical volume of vehicle is injected in contralateral hind footpad. Animals are sacrificed on day 7 and lymph nodes removed and weighed. The typical parameter of the PLNA is the weight index calculated from the mean weight ratio of lymph nodes draining from the treated footpad and the contralateral lymph node. Other parameters include cellularity index, measured as the ratio of cell counts from treated to control PLNs, histological examination, flow-cytometry analysis (Descotes, 1992).

PLNA suffers from various drawbacks such as evidence of false positives and the inability of assay to provide mechanistic information (Korte et al., 1991; Pieters, 2001; Descotes et al.,

1997). Various modifications of the PLNA have therefore been proposed (Albers et al., 1997; Lee et al., 2002).

1.2.4 Mouse ear swelling test (MEST)

MEST, the first predictive method based upon challenge-induced increase in ear thickness in previously sensitised mice, was described by Gad and colleagues (Gad et al., 1986). The MEST involved induction of sensitisation, comprising recurring four consecutive daily applications of the test chemical on tape-stripped abdominal skin, the site prepared previously with an intradermal injection of adjuvant to increase immunogenicity. One week following completion of the induction, both test and control mice are treated on the dorsum of one ear with the test chemical and on the contralateral ear with vehicle. Changes in ear thickness are measured 24 and 48 hours following challenge.

This method is tedious and painstaking involving tape stripping. Various investigators recommended modifications in the MEST protocol, including a vitamin A enriched diet for enhancing cell-mediated immune function and contact and delayed-type hypersensitivity responses (Maisey and Miller, 1986), replacing the adjuvant treatment and tape stripping for the purposes of increasing the efficiency of sensitisation (Thorne et al., 1991).

1.2.5 Local Lymph Node Assay (LLNA)

The LLNA is based upon consideration of the immunobiological events that are stimulated during the induction phase of skin sensitisation (Kimber at al., 1986). It has been subjected to both national and international inter-laboratory collaborative trials. LLNA offers a number of important advantages compared with other animal methods. The assay is objective, not relying on the assessment of induced erythematous reactions, and is not subject to interpretative difficulties when coloured materials are examined. The test does not require the use of adjuvant, epidermal tape stripping or dietary supplements and exposure to chemical is via the relevant route. There are advantages also in terms of animal welfare considerations; fewer animals are needed and the trauma to which animals are potentially subject is reduced.

Briefly, the standard assay is performed as follows. Groups of mice (CBA/Ca strain) are exposed daily, for 3 consecutive days, to various concentrations of the test material, or to an

equal volume of the relevant vehicle alone, on the dorsum of both ears. Five days following the initiation of exposure animals are injected intravenously with ^{3}H-thymidine. Mice are sacrificed 5 hours later and the draining (auricular) lymph nodes are isolated and pooled for each experimental group. Single-cell suspensions of LNC are prepared by mechanical disaggregation and processed for liquid scintillation counting. Results are recorded as disintegrations per minute per lymph node for each experimental group. From these values a stimulation index for each concentration of test material is derived relative to vehicle controls.

The criterion for a positive response in the LLNA, and for classification of a chemical as a sensitiser, is that at one or more concentrations of the test material a stimulation index of three or greater is elicited. The effective concentration required for a three-fold increase in LNC proliferative capacity compared with concurrent vehicle controls (EC3 value), has already proven to be an effective means of assessing the relative skin sensitising potency of chemicals (Basketter et al., 1997; Hilton et al., 1998).

In 1999, LLNA was accepted by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 1999) and by the European Centre for the Validation of Alternative Methods (Balls and Hellsten, 2000). It has been accepted as being a stand-alone method for screening chemicals for skin sensitising activity as the first step in an assessment process (CPMP, 2001; FDA, 2002; OECD, 2002; EPA 2003). For skin sensitisation testing, REACH specifies that the “LLNA is the first-choice method for *in vivo* testing. Only in exceptional circumstances should another test be used. Justification for the use of another test shall be provided” (EC 2006).

1.2.5.1 Alternative variants of LLNA

Various investigators have used alternative species such as rats, guinea pigs and hamsters in variants of LLNA (Ikarashi et al., 1992; Clottens et al., 1996; Arts et al., 1997). Although the ICCVAM guideline recommends use of CBA mice only, different strains of mice have been studied in the LLNA by Woolhiser and coworkers (2000), who proposed that DBA/2, B6C3F1 and BALB/c are good alternatives as additional mouse strains for use in LLNA. In addition, different working groups have investigated alternative endpoints and alternative treatment protocols (Ikarashi et al., 1992 and 1993, Suda et al., 2002; Picotti et al., 2006). In an effort to avoid the use of radioisotopes, Takeyoshi and coworkers replaced radioactive [^{3}H]thymidine by 5-bromo-2-deoxyuridine (BrdU) (Takeyoshi et al., 2001). A method based on measurement of IL-2 released in the supernatant of the lymph node cell culture following

in vivo treatment of the mice was proposed by Hatao et al., (1995). Ulrich and Vohr have investigated various cytokines as endpoints in the LLNA (Ulrich and Vohr, 1996; Ulrich et al., 1998).

The drawbacks with the standard LLNA protocol is the use of radioisotopes and the concern about a possible impact of irritant properties of the test chemical or other non-specific activation of immune cells which may cause non-specific cell proliferation in the draining lymph node and thus lead to false positive results (Montelius et al., 1994, 1998; Basketter et al., 1998; Loveless et al., 1996; Vohr et al., 2000; Vohr and Ahr, 2005; Ulrich et al., 2001).

Homey and coworkers put forward a protocol involving measurement of the ear and lymph node parameters in order to differentiate between chemical-induced allergic and irritant skin reactions (Homey et al., 1997; 1998). A biphasic protocol was proposed by Ulrich and coworkers (Ulrich et al., 2001), involving treating the mice with the chemical on the shaved back followed by treatment on the ear after 12 days, and measuring responses in the ear skin and the ear-draining lymph node, which included ear weight, lymph node weight and lymph node cell count. The draining lymph node and ear end-points were also used by other investigators, and found useful for the purpose (Lee et al., 2002; Ehling et al., 2005a,b; Suda et al., 2002; Gamer et al., 2008).

Several authors have introduced a flow cytometric evaluation of activated cells in the ear draining lymph nodes (Homey et al., 1997 and 1998; Takeyoshi et al., 2001; Yamashita et al., 2005; Gerberick et al., 1999 and 2002), which can provide mechanistic information.

The present investigation is based on the biphasic protocol of the LLNA. The mice were treated in two phases, the first being the sensitisation phase and the second is the challenge phase. One day after the last treatment, the mice were killed and the end-points evaluated. The assay offers the advantages being simple, objective and having quantitative endpoints. This protocol has also been used earlier in our laboratory (Stahlmann et al. 2006; Ahuja et al., 2009 a,b), the results showing that the biphasic sensitisation-challenge protocol with an induction and an elicitation phase, has higher sensitivity and specificity than the commonly used sensitisation protocol. It allowed us to identify weak sensitisers more precisely.

1.3 In vitro models

1.3.1 Keratinocyte culture

More than 90% of the cells in the epidermis are keratinocytes (KCs) and, are the first cells to come across chemicals that penetrate through the stratum corneum of the skin. KCs secrete a variety of proinflammatory cytokines, chemokines and growth factors (Matsue et al., 1992), which play important role in ACD (Schwarz and Luger, 1992). The relative ease of KC culture makes them an interesting object for the development of *in vitro* methods for predictive contact sensitisation testing. However, KCs are not true 'immune' cells and can not differentiate allergens from irritants.

While working with epidermal cell cultures, Coutant et al. (1999) reported that the strong contact allergen trinitrobenzene sulfonic acid (TNBS) induced CD40 expression on mice KCs, whereas the irritant SLS did not. Wakem et al. (2000) demonstrated that CD80 expression in human KCs was upregulated to similar extents by treatment with either allergens or irritants. This proves that KCs respond to a toxic insult only and are not able to differentiate allergens from irritants.

1.3.2 Langerhans cell cultures

Langerhans cells (LCs), the major antigen presenting cells (APCs) in the skin, play an important role in the development of allergic contact sensitisation. LCs comprise 1-3% of epidermal cells. Various isolation techniques have been developed to acquire LCs from human and murine sources (Hanau et al., 1988; Teunissen et al., 1988; Simon et al., 1995), but the amount of cells obtained is relatively low. Also, no LCs cell-line has been established so far. Therefore, the availability of adequate cells is a limiting factor in the development of *in vitro* methods based on LCs.

Verrier et al. (1999) showed modulation of E-cadherin and HLA-DR expression on human LCs exposed to contact sensitizers, while there was no effect of treatment with an irritant. Freshly isolated human LCs did not show any changes in phosphotyrosine (p-tyr) following exposure to strong hapten MCI/MI, while LCs cultured for 24 hours demonstrated a significant increase in p-tyr (Kühn et al., 1998).

1.3.3 Peripheral blood-derived dendritic cells

Dendritic cells (DCs) are a discrete group of leukocytes with the ability to initiate immune response by processing and presenting antigens. Langerhans cells were the first identified DCs, performing the sentinel role of immature DCs (Banchereau and Steinman, 1998). The development of techniques to generate DCs from CD34+ precursors, from bone marrow or cord blood, and peripheral blood mononuclear cells (PBMCs) has led to a source of LCs-like cells for study purposes.

Aiba et al. (1997) observed that haptens like $NiCl_2$ and DNCB cause significant enhancement in the expression of CD54, CD86 and HLA-DR on monocyte-derived DCs, while no such changes were seen in DCs treated with irritants. However, a donor-to-donor variability was observed in changes in expression of the markers, with some subjects showing no changes. They also found that DCs incubated with $NiCl_2$ showed significant augmentation of IL-1β, IL-6 and TNF-α, while exposure to DNCB induced increased secretion of IL-1β alone. Coutant et al. (1999) demonstrated that monocyte-derived DCs expressed increased levels of HLA-DR, CD86, CD40 and CD54 in the presence of haptens in comparison to irritants. Following incubation of DCs with haptens, they observed increased levels of TNF-α than in comparison to irritants.

DCs from peripheral blood can be obtained in adequate quantities; a DC-based approach therefore being a promising effort for developing an *in vitro* method to envisage the sensitisation potential of chemicals. However, the variability in donor-to-donor responses should be taken care of.

1.3.4 Human skin equivalent/reconstituted epidermis cultures

Three-dimensional *in vitro* cultures of human skin are of two types: epidermal or skin equivalents. Epidermal equivalents contain keratinocytes, which are cultured on a filter or matrix at the air-liquid interface, developing into a completely differentiated epidermis with a stratum corneum. Various epidermal equivalent cultures are commercially available, EpiDerm™ from MatTek Corporation, Reconstructed Human Epidermis from Skinethic (Rosdy, 1994) and Episkin™ from IMEDEX (Tinois et al., 1994). Skin equivalents involve epidermis and a dermal equivalent, consisting of either de-epidermized human epidermis or dermal substitutes such as collagen or nylon mesh matrices containing fibroblasts (Regnier et al., 1990).

Gerberick and Sikorski (1998) concluded that the cytokine levels in skin equivalents could be modulated following exposure to chemicals, however, the changes were found to be exclusive for the chemical and were also concentration and or time dependent. No single cytokine or profile of cytokines could be recognized as predictive for sensitisation potential. However, Corsini et al. (1999) found that IL-12 was increased in the Episkin model following treatment with chemical allergens. Regnier et al. (1997) and Fransson et al. (1998) have introduced LCs into skin equivalent cultures.

Three-dimensional models provide advantages over monolayer cultures; having differentiated stratum corneum and an air-liquid interface, which permits use of nonaqueous compatible test materials.

1.3.5 Human skin explant cultures

Skin explant cultures involve full-thickness human skin obtained from breast or abdominal reduction surgery. Explants contain immune cells including LCs, along with other skin cells such as melanocytes and endothelial cells, in addition to keratinocytes and fibroblasts. Rambukkana et al. (1996) studied changes in surface marker and cytokine expression by LCs in the skin explants. They found that following exposure to allergens, the LCs which migrated towards the epidermal-dermal junction expressed decreased levels of CD1a and HLA-DR along with a significant increase of ICAM-1 (CD54).

Although explant cultures resemble more to *in vivo* skin in structure and cell population, they also have drawbacks (Ponec, 1992). Skin organ cultures can be maintained *in vitro* only for a short time, since the cultured cells either migrate or grow out of the skin. The availability of fresh tissue is also limited and the degree of variability from explant to explant and individual to individual is high.

1.3.6 Co-culture systems

Since the initiation of ACD involves interaction between LCs and T-cells, a system containing both, would be a preferable approach as an *in vitro* method for sensitisation testing of chemicals. Using DCs obtained from CD34+ cord blood cells, Rougier et al. (1998 and 2000)

found that strong allergens led to significant proliferation of naïve autologus lymphocytes, in comparison to the irritants.

Use of peripheral blood-derived DCs and autologus T-cells from human blood donors as an *in vitro* method for testing sensitisation potential provides a simple method. However, there is a possibility of significant donor-to-donor variability in the response.

The present *in vitro* study was performed using a co-culture model of keratinocytes and monocytes, the loose-fit coculture based sensitisation assay (LCSA). This assay was initially developed by Wanner and coworkers (Schreiner et al., 2007 and 2008), and was found to be suitable enough for the testing of sensitisation potential of chemicals. The advantages of the LCSA include: assessment of dose-response information, easy to perform, reproducible, donor-variance being negligible. The present investigation was performed in collaboration with the developers of the assay.

1.4 Objectives of the study:

The present study was performed with the following objectives:

1. To study the suitability of the biphasic treatment protocol and the ear and draining lymph node end-points in the modified local lymph node assay for assessing the sensitising potential of various textile dyes

2. To study the appropriateness of studying the cell-surface markers with the help of flow-cytometry for investigating the sensitising potential of textile dyes using the biphasic mice local lymph node assay.

3. To study the dose-response effect of various disperse dyes using a biphasic modification of the mice local lymph node assay and to characterise the differences in potency of the dyes to induce sensitisation.

4. To study the sensitisation potency of various disperse dyes using an *in vitro* loose-fit coculture based sensitisation assay (LCSA).

5. To compare the results of the LLNA and LCSA studies in an effort to assess the suitability of the *in vitro* method for assessing the sensitising potency of chemicals for risk assessment.

2 Material and Methods

2.1 Textile Dyes

The textile dyes were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Detailed information about the investigated textile dyes is given in Table 2.1.

No.	Textile dye	CAS No.	Mol. Wt.	Product No.	Molecular Formula
1.	Disperse blue 1	2475-45-8	268.27	215643	$C_{14}H_{12}N_4O_2$
2.	Disperse blue 35	12222-75-2	284.10	17992	$C_{15}H_{12}N_2O_4$
3.	Disperse blue 106	68516-81-4	335.38	28241	$C_{14}H_{17}N_5O_3S$
4.	Disperse blue 124	61951-51-7	377.42	21620	$C_{14}H_{17}N_5O_4S$
5.	Disperse yellow 3	2832-40-8	269.30	28225	$C_{15}H_{15}N_3O_2$
6.	Disperse orange 3	730-40-5	242.23	17983	$O_2NC_6H_4N{=}NC_6H_4NH_2$
7.	Disperse orange 37	13301-61-6	392.24	21603	$C_{17}H_{15}C_{l2}N_5O_2$
8.	Disperse Red 1	2872-52-8	314.34	344206	$C_{16}H_{18}N_4O_3$

Table 2.1 Detailed information about the textile dyes investigated in the study

2.2 Local lymph node assay

2.2.1 Animal maintenance

Six week old, female BALB/c mice were received from Forschungseinrichtung für experimentelle Medizin, Campus Benjamin Franklin, Charité Universitätsmedizin, Berlin. The mice were maintained in the animal house of the Department of Toxicology, Institute of Clinical Pharmacology and Toxicology, Charité Universitätsmedizin, Campus Benjamin Franklin, Berlin. The mice were acclimatised for one week before the start of the experiment. The animals weighed between 18 to 22 g. Five animals were grouped together in plastic (Macrolon® type 4) cages. The room temperature was maintained at 23±1°C, and the relative humidity was kept at 45–55%. The animals were maintained in artificial lighting, with 12 hr each of light and dark cycle (light phase between 9:00 and 21:00 hr). Pellet feed (Pellet feed 1324, Altromin) and tap water were given ad libitum.

The experiments were performed in accordance with permission (No.: G0047/08) from Landesamt für Gesundheit und Soziales Berlin (LAGeSo, Berlin).

2.2.2 Treatment of animals

The animals were shaved over a surface of approximately 2 cm^2 on their back, and treated using a “biphasic” or “sensitisation-challenge protocol”. Solutions were prepared freshly for each application in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany). Ten animals were used for each concentration of the textile dye tested, while 40 animals were used in total for the vehicle treated control group.

Day 1 to 3 "Sensitisation phase": To sensitize the animal, the shaved area on the back was treated once daily on days 1 to 3 with 50µl of the test solution in DMSO, with the concentrations mentioned in Table 2.2. All mice remained untreated on days 4 to 14 (Fig. 2.1). The control animals were treated with the vehicle alone, with the same protocol as for the treated animals.

The dose selection for various dyes was done on the basis of their solubility and their sensitisation potential after reviewing earlier studies (Hausen and Sawall, 1989; Hausen and Brandao, 1986; Betts et al., 2005).

Day 15 to 17 "Challenge phase": The treatment was repeated with 25µl of the solution applied once a day for 3 consecutive days on days 15 to 17 on the dorsum of both ears.

Day 19 "End-point analysis": The animals were euthanized with deep CO_2 anaesthesia, lymph nodes prepared, and end-points analysed.

The animal handling was always performed at 11:00 am, by the same person.

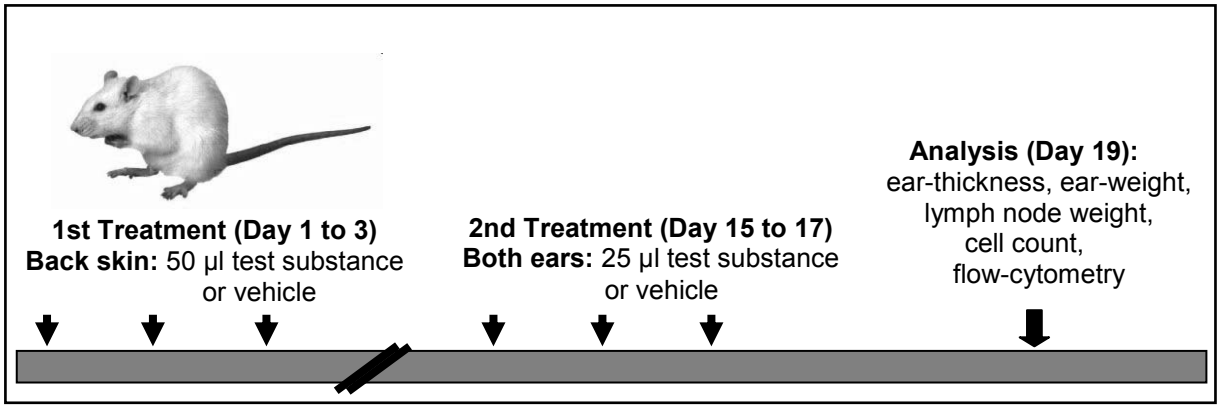


Fig. 2.1 Pictorial representation of the biphasic protocol of the local lymph node assay

Textile dye	Tested concentrations in LLNA
Disperse blue 1	10 and 3%
Disperse blue 35	30 and 10%
Disperse blue 106	30, 3, 0.3, 0.03 and 0.003%
Disperse blue 124	10, 3, 0.3, 0.03 and 0.003%
Disperse yellow 3	30 and 10%
Disperse orange 3	30%
Disperse orange 37	30 and 10%
Disperse Red 1	30, 10 and 3%

Table 2.2 Concentrations of various textile dyes investigated in LLNA for assessing their sensitisation potential

2.2.3 Experimental procedure

The animals were euthanized with deep CO_2 anaesthesia in a closed chamber. The dead animal was then taken and ear thickness, ear-punch weight, lymph node weight and lymph node cell count analysed.

2.2.3.1 Measurement of ear thickness

The ear thickness (mm) was measured with a spring-loaded micrometer, Oditest (Kroeplin, Schüchtern, Germany). Care was taken to always measure the thickness from 2 mm inside of the outer periphery of the ear. Each measurement was repeated thrice and the average value was recorded.

2.2.3.2 Measurement of ear-punch weight

A section was taken from both ears with a punch of 6 mm diameter and weighed (mg). Care was taken to take the biopsy from 2 mm inside the outer periphery of the ear. Any extra tissue or hairs, if present, were removed carefully before weighing.

2.2.3.3 Measurement of lymph node weight

For removing the lymph node, the mouse was fixed in a ventral position on a dissection table. With the mouse ventrally exposed, the neck and abdomen area was wetted with 70% ethanol. Using scissors and forceps, a first incision was made carefully across the chest and between the forelegs. Care was taken to prevent any incision near to the heart. A second incision was made up the midline, perpendicular to the initial cut, and then cut up to the chin area. The skin was reflected to expose the external jugular veins in the neck area. Care was taken to avoid causing any damage to the salivary tissue at the midline and nodes associated with this tissue, and also the jugular vein. The nodes draining the ear (“auricular”) are located distal to the masseter muscle, away from the midline, and near the bifurcation of the jugular veins (Fig 2.2). The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. The draining auricular lymph nodes were excised carefully, excessive tissue removed, and weighed (mg).

2.2.3.4 Measurement of lymph node cell count

The lymph nodes were placed in 2 ml phosphate buffered saline (PBS, Biochrom, Berlin, Germany). A single cell suspension from each lymph node was prepared by gentle mechanical disaggregation by a forcep and then filtering through stainless-steel mesh filter.

The cell-count (million/ml) was then measured using an automated cell counter Sysmex F 820 (Sysmex Europe GmbH, Norderstedt, Germany).

The automated cell counter makes a dilution of the sample, which is then added with 2-3 drops of Quicklyser II (Medical electronics Co.; Kobe, Japan) in order to dissolve the cell membrane of lymphocytes, so that the countable lymphocyte nuclei are released. The detection of cell number is based on physical measurement of electrical resistance within a capillary. The diluent of the device has a defined electrical resistance which passes along with the lysed lymphocytes across a measuring electrode in the capillary. When a lymphocyte cell nucleus comes across in the capillary, it changes the electrical resistance within the capillary and the device counts this event as a cell. The device adds up the number of events and calculates the cell count in millions / ml of solution.

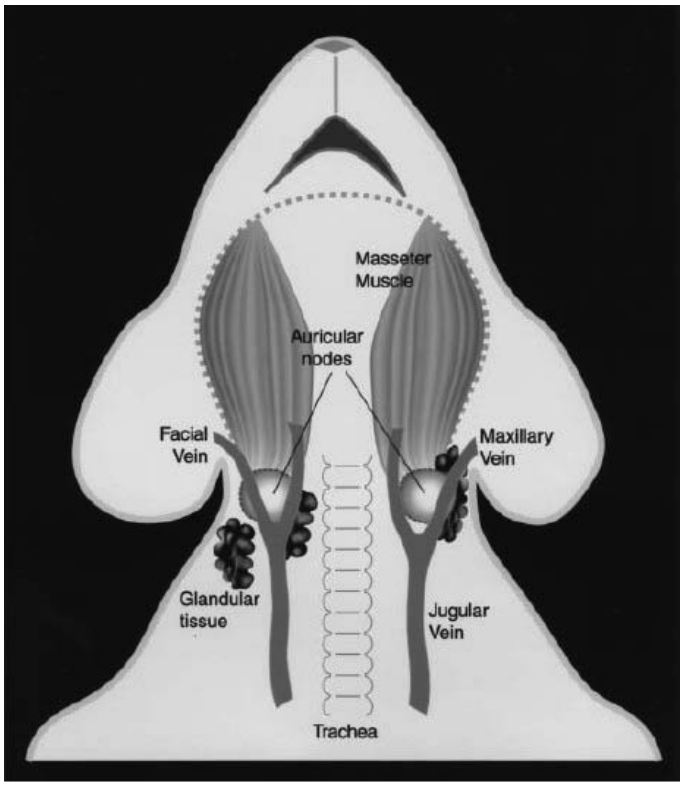


Fig. 2.2 Position of auricular lymph node in mouse dissected ventrally (Dean et al., 2001)

2.2.3.5 Immunophenotyping of the cells

The cells were marked with antibodies for various lymphocyte surface markers and then measured with a flow cytometer. The antibodies and isotype control used in the investigation are tabulated in Table 2.3, and were purchased from BD Biosciences (BD Pharmingen™, Heidelberg, Germany)

Briefly, cells (0.5 million) were taken in 5ml FACS tubes (BD Falcon™, Ref 352052), added with corresponding antibodies, and incubated at 4°C for 30 min in the dark. For every lymph node, five samples were prepared for measuring, as shown in the Table 2.4. Unmarked cells and cells incubated with the isotype control were also kept for adjusting the FACS settings and compensation.

Antibody (Catalog no.)	Clone	Isotype control (Catalog no.)
FITC Rat Anti-Mouse CD4 (553651)	H129.19	Rat IgG2a, $_K$ (553929)
PE Rat Anti-Mouse CD4 (553049)	RM4-5	Rat IgG2a, $_K$ (553930)
PE Rat Anti-Mouse CD8 (553033)	53-6.7	Rat IgG2a, $_K$ (553930)
PE Rat Anti-Mouse CD19 (553786)	1D3	Rat IgG2a, $_K$ (553930)
FITC Rat Anti-Mouse CD45R/B220 (553088)	RA3-6B2	Rat IgG2a, $_K$ (553929)
FITC Hamster Anti-Mouse CD69 (553236)	H1.2F3	Hamster IgG1, λ1 (553953)
PE Rat Anti Mouse1-A/1-E (557000)	M5/114.15.2	Rat IgG2b, $_K$ (553989)

Table 2.3 Fluorochrome conjugated antibodies and corresponding isotypes used for FACS in LLNA

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
FITC	CD4	-	CD45	CD45	CD69
PE	CD8	CD19	-	1A	CD4

Table 2.4 Different samples prepared from single lymph node using various antibodies

After the incubation period, the cells were washed twice with a solution of 0.05% sodium azide (Sigma-Aldrich Chemie) in PBS. The cells were then measured by FACS Calibur flow cytometer (Becton Dickinson). 1×10^4 lymphocytes were counted per sample.

2.2.3.6 Flow cytometry

Flow cytometry is a technique that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records the way in which the cell or particle scatters incident laser light and emits fluorescence.

A flow cytometer is made up of three main systems: fluidics, optics, and electronics (Fig 2.2).

- The fluidics system transports particles in a stream to the laser beam for interrogation.

- The optics system consists of lasers to illuminate the particles in the fluid stream and optical filters to direct the resulting light signals to the appropriate detectors.

- The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2-150 micrometers in size is suitable for analysis. When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecules present on the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters diverts the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them. List mode data are collected on each particle or event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer. This data can be analyzed to provide information about subpopulations within the sample.

2.2.3.6.1 Fluidics

The purpose of the fluidics system is to transport particles in a fluid stream to the laser beam for interrogation. For optimal illumination, the stream transporting the particles should be positioned in the center of the laser beam. In addition, only one cell or particle should move through the laser beam at a given moment.

To accomplish this, the sample is injected into a stream of sheath fluid within the flow chamber. The design of the flow chamber causes the sample core to be focused in the center of the sheath fluid where the laser beam will then interact with the particles. Based on principles relating to laminar flow, the sample core remains separate but coaxial within the sheath fluid. The flow of sheath fluid accelerates the particles and restricts them to the center of the sample core. This process is known as hydrodynamic focusing.

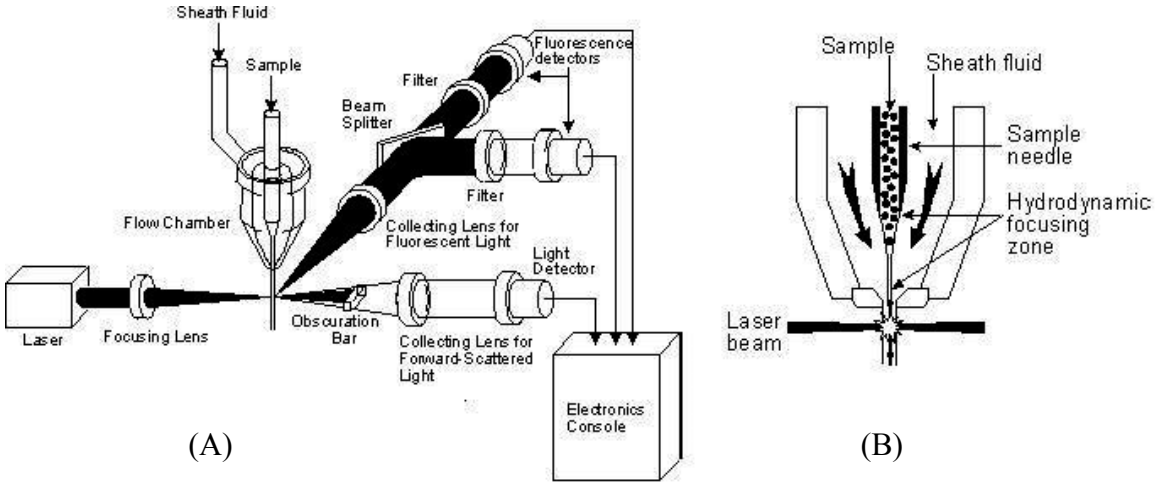


Fig. 2.3 Schematic view of a flow cytometer (A) flow chamber (B) [Source: http://www.ibot.cas.cz/fcm/instr.html]

2.2.3.6.2 Light Scatter

Light scattering occurs when a particle deflects incident laser light. The extent to which this occurs depends on the physical properties of a particle, namely its size and internal complexity. Factors that affect light scattering are the cell's membrane, nucleus, and any granular material inside the cell. Cell shape and surface topography also contribute to the total light scatter.

Forward-scattered light (FSC) is proportional to cell-surface area or size. FSC is a measurement of mostly diffracted light and is detected by a photodiode. FSC provides a suitable method of detecting particles greater than a given size independent of their fluorescence and is therefore often used in immunophenotyping to trigger signal processing.

Side-scattered light (SSC) is proportional to cell granularity or internal complexity. SSC is a measurement of mostly refracted and reflected light that occurs at any interface within the cell where there is a change in refractive index.

Correlated measurements of FSC and SSC can allow for differentiation of cell types in a heterogeneous cell population.

2.2.3.6.3 Fluorescence

A fluorescent compound absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level. The excited electron quickly decays to its ground state, emitting the excess energy as a photon of light. This transition of energy is called fluorescence.

The range over which a fluorescent compound can be excited is termed its absorption spectrum. As more energy is consumed in absorption transitions than is emitted in fluorescent transitions, emitted wavelengths will be longer than those absorbed. The range of emitted wavelengths for a particular compound is termed its emission spectrum.

The argon ion laser is commonly used in flow cytometry because the 488-nm light that it emits excites more than one fluorochrome. One of these fluorochromes is fluorescein isothiocyanate (FITC). In the absorption spectrum of FITC, the 488-nm line is close to the FITC absorption maximum. Excitation with this wavelength will result in a high FITC emission. If the fluorochrome were excited by another wavelength within its absorption spectrum, light emission of the same spectrum would occur but it would not be of the same intensity.

More than one fluorochrome can be used simultaneously if each is excited at 488 nm and if the peak emission wavelengths are not extremely close to each other. The combination of FITC and phycoerythrin (PE) satisfies these criteria. Although the absorption maximum of PE is not at 488 nm, the fluorochrome is excited enough at this wavelength to provide adequate fluorescence emission for detection. More important, the peak emission wavelength is 530 nm for FITC and 570 nm for PE. These peak emission wavelengths are far enough apart so that each signal can be detected by a separate detector. The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the particle.

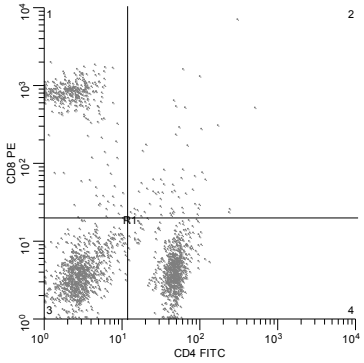
2.2.3.6.4 Computer analysis

The data from the detectors is sent to a computer and plotted as a dot plot or histogram, which can be further used for analysis.

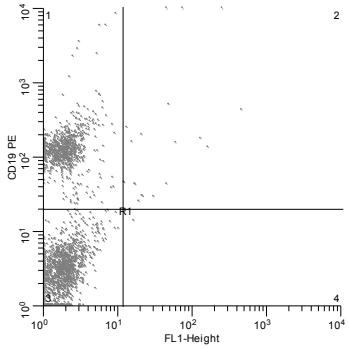
2.2.3.7 Data analysis

Mean values were calculated for the ear thickness, ear-punch weight, lymph node cellularity, lymph node weight and lymphocyte sub-population of each animal. Results from the mice treated with the dye solutions were compared to those obtained from vehicle treated control animals using t-test. Statistical analysis was done with the software SPSS 16.0 (SPSS Inc., Chicago, USA).

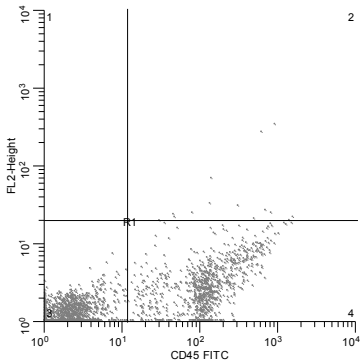
FACS data were analysed using Winlist 5.0 software (Verity Software House, Topsham, USA) and statistically analysed using t-test with SPSS 16.0. The various cell populations were analysed in our study as shown in Fig. 2.3



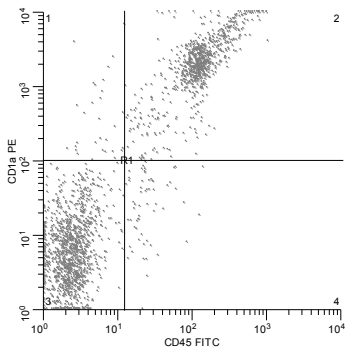
(i) CD4+ and CD8+ cells



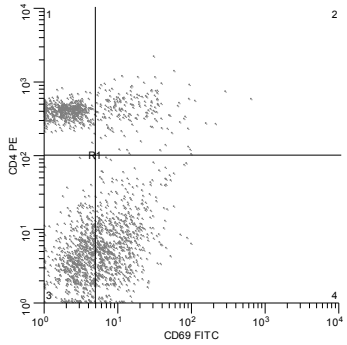
(ii) CD19+ cells



(iii) CD45+ cells



(iv) CD45+/1A+ cells



(v) CD69+/CD4+ cells

Fig. 2.4 Examples of FACS data analysis showing subpopulations of lymphocytes analysed in LLNA

2.3 The “loose-fit coculture-based sensitisation assay”

The “loose-fit coculture-based sensitisation assay (LCSA)” was developed by Wanner and coworkers (Schreiner et al., 2007, 2008). The present study was carried out in collaboration with the developers of this assay, using the original protocol.

2.3.1 Media and reagents

The cells were cultured in serum-free keratinocyte growth medium-2 (KGM-2; PromoCell, Heidelberg, Germany) added with 100 U/ml penicillin and 100μg/ml streptomycin (both: Biochrom, Berlin, Germany) and Supplement Pack/ Keratinocyte Growth Medium 2 (PromoCell) which contains BPE-15, hEGF-0.0625, HC-165, Insulin-2.5, Epinephrine-195, Transferrin-5, $CaCl_2$-0.5/0.15. The textile dyes were dissolved in dimethyl sulfoxide (DMSO; Hybrimax, Sigma). Care was taken that the final concentrations of DMSO in culture media did not exceed 0.2%.

2.3.2 Cryopreservation of primary human keratinocytes

Human skin was received from healthy donors in accordance to the Declaration of Helsinki principles as left-over material from plastic surgery with local ethics committee approval. Following incubation in PBS containing 2 U/ml of dispase I (Roche, Mannheim, Germany) for

18 h at 4°C, epidermal sheets were stripped off the dermal layer and dissociated in PBS containing 0.25% trypsin (Biochrom) and 0.01% DNase (Roche) for 15min at 37°C. Single-cell suspension was obtained by passing through a 40μm cell strainer (BD Biosciences). Cells were washed in PBS, resuspended in KGM-2, and seeded on Costar Cell Culture Flasks (Corning, Schiphol-Rijk, The Netherlands) at a density of 2–5×10^5 cells/cm^2. The cells were cultured until confluence changing medium on alternate days. The keratinocytes were harvested by trypsinization and cryopreserved in fetal calf serum (Biochrom) with 10% DMSO at a density of 6×10^6 cells/ml.

2.3.3 Cryopreservation of human monocytes

Peripheral blood mononuclear cells (PBMC) were enriched from fresh buffy coat preparations (German Red Cross, Berlin) by density centrifugation on Ficoll-Paque (Biochrom). Monocytes were isolated from PBMCs CD14+ magnetic cell sorting using magnetically labeled anti-CD14 antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany). Monocytes were frozen at a concentration of 2–3×10^7 cells/ml in fetal calf serum (Biochrom) with 10% DMSO.

2.3.4 Loose-fit coculture-based sensitization assay (LCSA)

Cryopreserved keratinocytes were taken, thawed quickly, washed twice with PBS to remove any remaining DMSO and seeded in 12-well plates (Costar Cell Culture Cluster, Corning) at a density of 2–6×10^4 cells/cm^2 in serum-free KGM-2 medium. The cryopreserved allogenic CD14+ monocytes were also thawed and washed in the same manner as for keratinocytes, followed by suspending them into the medium. After about 1.5 hour, when half-confluence was reached for keratinocytes, the medium from the wells in the plate is withdrawn to remove any dead or floating cells. Monocytes were seeded onto keratinocytes at a density of 2–5×10^5 cells/cm^2, followed by addition of IL-4, GM-CSF (both from Immunotools, Friesoythe, Germany) and TGF-β1 (R&D, Wiesbaden-Nordenstadt, Germany) at final concentrations of 100, 100, and 10 ng/ml, respectively. After 2 days of generation period, textile dyes were added. After 48 h, non-adherent cells were pipetted off the coculture, counted (CoulterZ1, Beckman Coulter) and prepared for FACS analysis.

2.3.5 FACS analysis

Cells were analysed using FACS Calibur flow cytometer (BD Biosciences). Uptake of 7-amino-actinomycin (7-AAD; ViaProbe, BD Biosciences) was used to determine/exclude dead cells. The conjugated antibodies used with their corresponding isotypes are tabulated in Table 2.5.

Antibody	Clone	Isotype	Company
FITC anti-CD11c	BU15	mouse IgG1	Serotec, Düsseldorf, Germany
FITC anti-CD1c	AD5-8E7	mouse IgG2a	Miltenyi Biotec
FITC anti-CD14	M5E2	mouse IgG2a	BD Biosciences
APC anti-HLA-DR	G46-6	mouse IgG2a	BD Biosciences
PE anti-CD86	FUN-1	mouse IgG1	BD Biosciences
APC anti-CD1a	HI149	mouse IgG1	BD Biosciences

Table 2.5 Fluorochrome conjugated antibodies and corresponding isotypes used for FACS in LCSA

Cells were gated to a distinct population of DCrc in scatter dot plots (Fig. 2.4). Relative upregulation of CD86 was determined as follows: MFI CD86 in treated cells/MFI CD86 in corresponding vehicle control. Only cocultures with pairs of the same donors during the same experiment were matched.

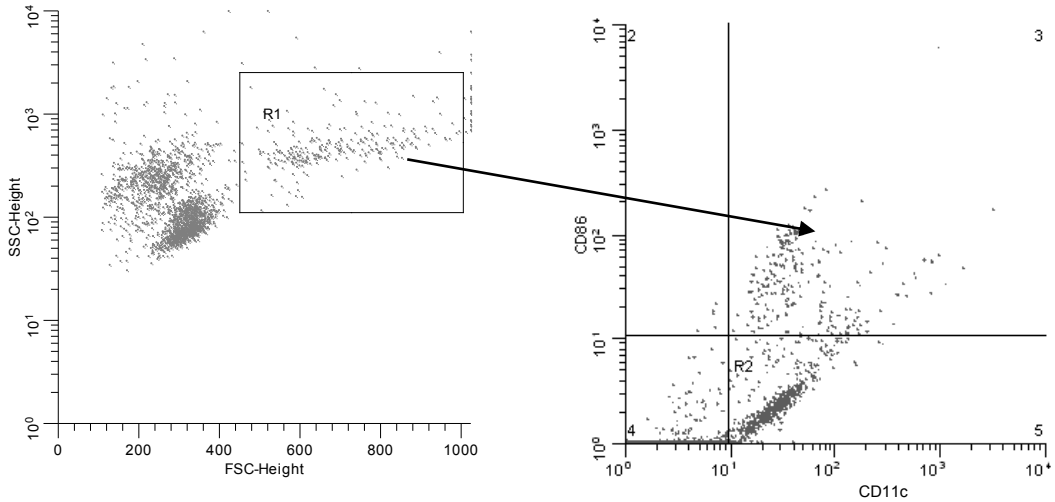


Fig. 2.5 For FACS analysis of CD86 expression, cells were gated in the region shown in scatter plot. The corresponding gated CD86+ cells are marked in the dot plot

3 Results

3.1 LLNA

3.1.1 Cell count measurement

Treatment with Disperse yellow 3 showed a significant increase (34%; change of the mean values are given i.e., mean value of treated group – mean value of control group / mean value of control group) in cell count at 30% concentration (Fig. 3.1.1.1). At 10% concentration of Disperse yellow 3, a non-significant (11%) increase in the cell-count was seen in the treated mice as compared to the control.

Disperse blue 124 treatment at 10, 3, 0.3, 0.03 and 0.003% concentrations showed significant increase in cell-count by 147, 132, 116, 79 and 21%, respectively, in the treated animals as compared to the control (Fig. 3.1.1.2)

A significant increase in the cell-count by 174, 124, 82, 79 and 37% was observed in mice following treatment with 30, 3, 0.3, 0.03 and 0.003 % concentrations, respectively, of Disperse blue 106 as compared to the control (Fig. 3.1.1.3).

Disperse orange 37 treatment at 30 and 10% concentrations showed significant increase (53 and 16%, respectively) in the cell count in the treated animals as compared to vehicle treated control animals (Fig. 3.1.1.4).

A significant modulation in the cell count (+61, +50 and +26%) was observed following Disperse red 1 treatment at 30, 10 and 3% concentrations, respectively, in the treated mice in comparison to vehicle control mice (Fig. 3.1.1.5).

Application of Disperse blue 35 at 30 and 10% concentrations showed significant enhancement in cell count by 32 and 24%, respectively, between the treated and control animals (Fig. 3.1.1.6).

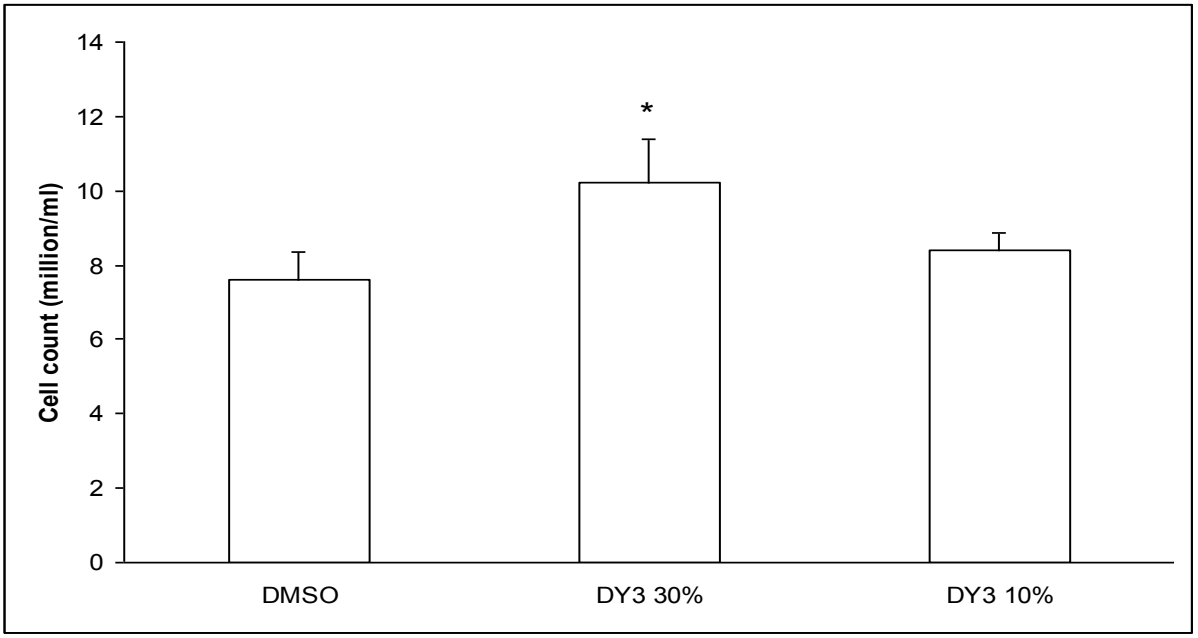


Fig. 3.1.1.1 Cell count (mean ± SD) values obtained from mice treated with Disperse Yellow 3 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

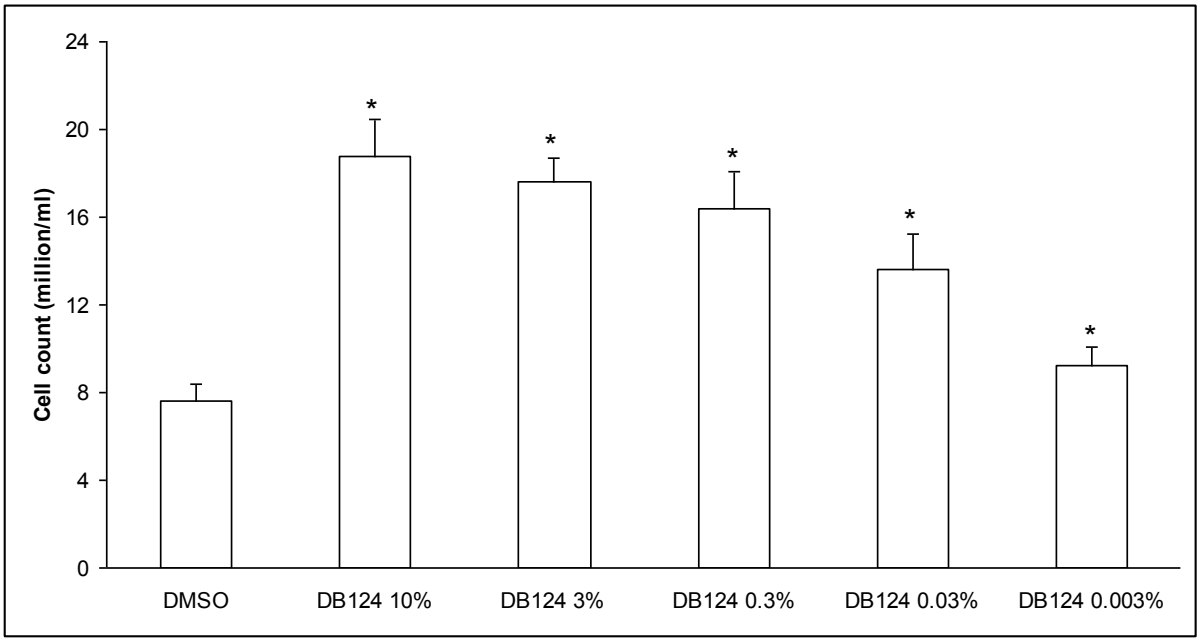


Fig. 3.1.1.2 Cell count (mean ± SD) values obtained from mice treated with Disperse Blue 124 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

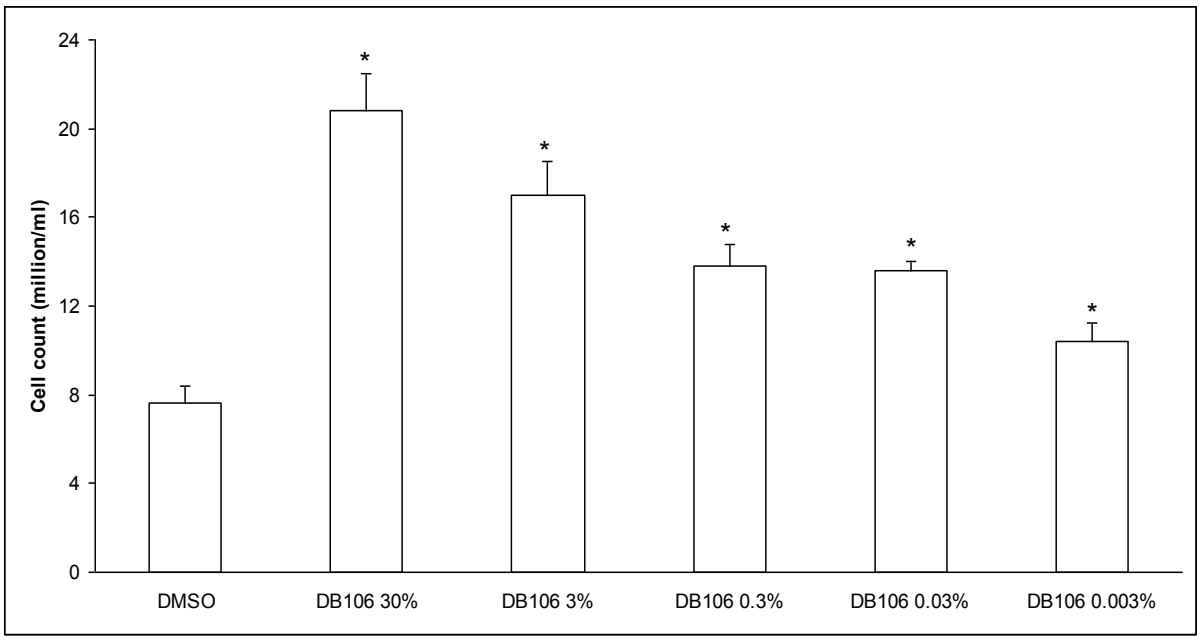


Fig. 3.1.1.3 Cell count (mean ± SD) values obtained from mice treated with Disperse Blue 106 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

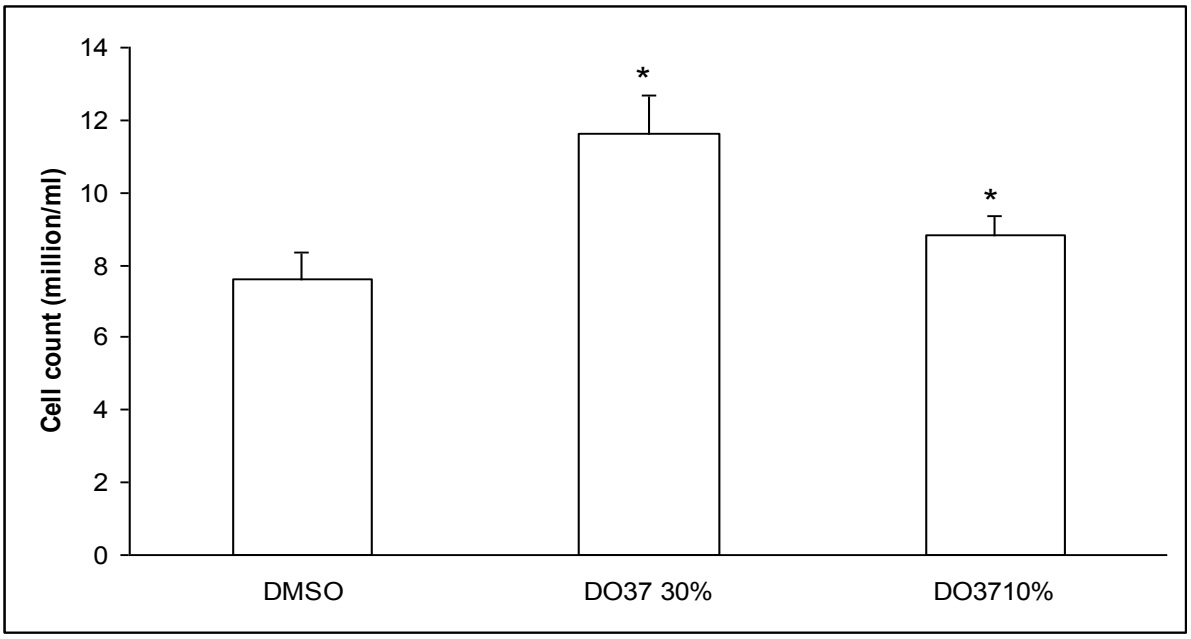


Fig. 3.1.1.4 Cell count (mean ± SD) values obtained from mice treated with Disperse Orange 37 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

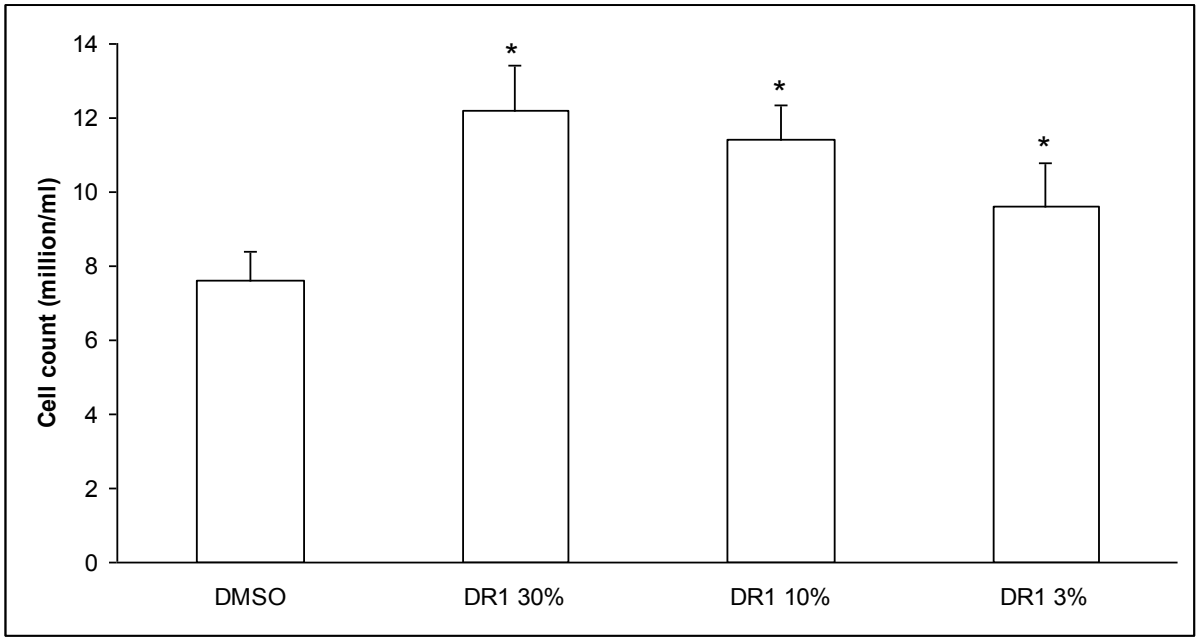


Fig. 3.1.1.5 Cell count (mean ± SD) values obtained from mice treated with Disperse Red 1 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

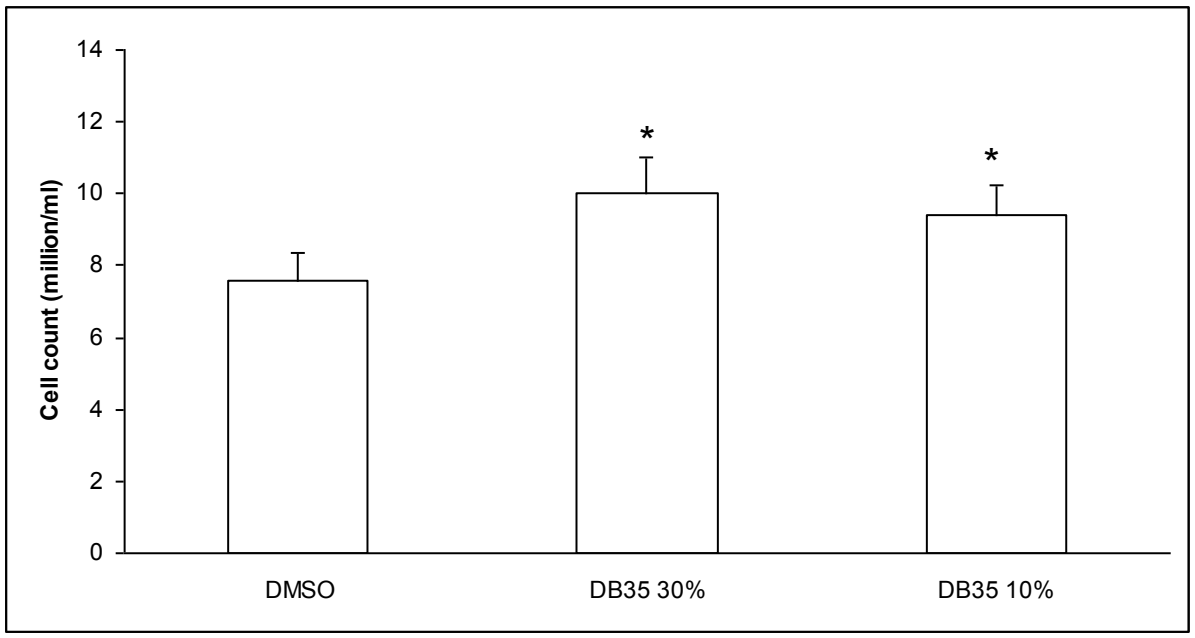


Fig. 3.1.1.6 Cell count (mean ± SD) values obtained from mice treated with Disperse Blue 35 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

In the experiment involving treatment of mice with Disperse orange 3 at 30% concentration, no difference was observed between the cell-count values of the treated and the control mice (Fig. 3.1.1.7).

In the investigation involving treatment with Disperse blue 1 at 10 and 3% concentrations, significant augmentation was observed in the cell count by 37 and 32%, respectively, between the treated and control animals (Fig. 3.1.1.8).

Based on our results, the disperse dyes could be arranged in four groups on the basis of their sensitising potency (Table 3.1.1.1) in the following decreasing order (in parenthesis: lowest concentration causing a significant increase in lymph node cell number): group 1, strong: Disperse blue 124 and Disperse blue 106 (0.003%), group 2, moderate: Disperse red 1 and Disperse blue 1 (3%), group 3, weak: Disperse orange 37 and Disperse blue 35 (10%) and group 4, very weak: Disperse yellow 3 and Disperse orange 3 (increase at 30% or no increase at 30%).

3.1.2 Lymph node weight measurement

A non-significant inflection in lymph node weight (+8%) was observed in the mice treated with Disperse yellow 3 at 30% concentration, as compared to the control animals (Fig. 3.1.2.1). At 10% concentration of Disperse yellow 3, the lymph node weight was found to increase significantly by 11%.

The lymph node weight was found to amplify significantly by 139, 125, 119, 86 and 25% following application of Disperse blue 124 at 10, 3, 0.3, 0.03 and 0.003% concentrations, respectively, in the treated mice as compared to the vehicle treated control mice (Fig. 3.1.2.2).

Treatment of mice with 30, 3, 0.3, 0.03 and 0.003% concentrations of Disperse blue 106 resulted in significant amplification of lymph node weight by 144, 106, 94, 78 and 28%, respectively, in the treated animals in comparison to the DMSO treated control animals (Fig. 3.1.2.3).

In the study involving application of Disperse orange 37 at 30 and 10% concentrations, a significant increase in the lymph node weight by 42 and 31%, respectively, was noticed in the treated mice as compared to the vehicle treated control mice (Fig. 3.1.2.4)

Significant increase in lymph node weight (53, 53 and 19%) was observed following treatment with 30, 10 and 3% solutions of Disperse red 1, respectively, in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.2.5)

Textile Dye	Concentration of textile dyes tested (%)						Lowest concentration causing significant increase in lymph node cell number (indicated in bold)	Classification for sensitising potency
	30	10	3.0	0.3	0.03	0.003		
DB 106	174	n.d.	124	82	79	**37**	0.003	strong
DB 124	nd	147	132	116	79	**21**	0.003	strong
DR 1	61	50	**26**	-	-	-	3.0	moderate
DB 1	-	37	**32**	-	-	-	3.0	moderate
DB 35	32	**24**	-	-	-	-	10.0	weak
DO 37	53	**16**	-	-	-	-	10.0	weak
DY 3	**34**	(11)*	-	-	-	-	30.0	very weak
DO 3	(30)*	-	-	-	-	-	> 30.0	very weak

*no significant increase at $p<0.05$ (t-test) between vehicle control and treated animals

Table 3.1.1.1 Increase in cell number (% of control) in lymph nodes of mice treated with various disperse dyes according to the sensitisation-challenge protocol

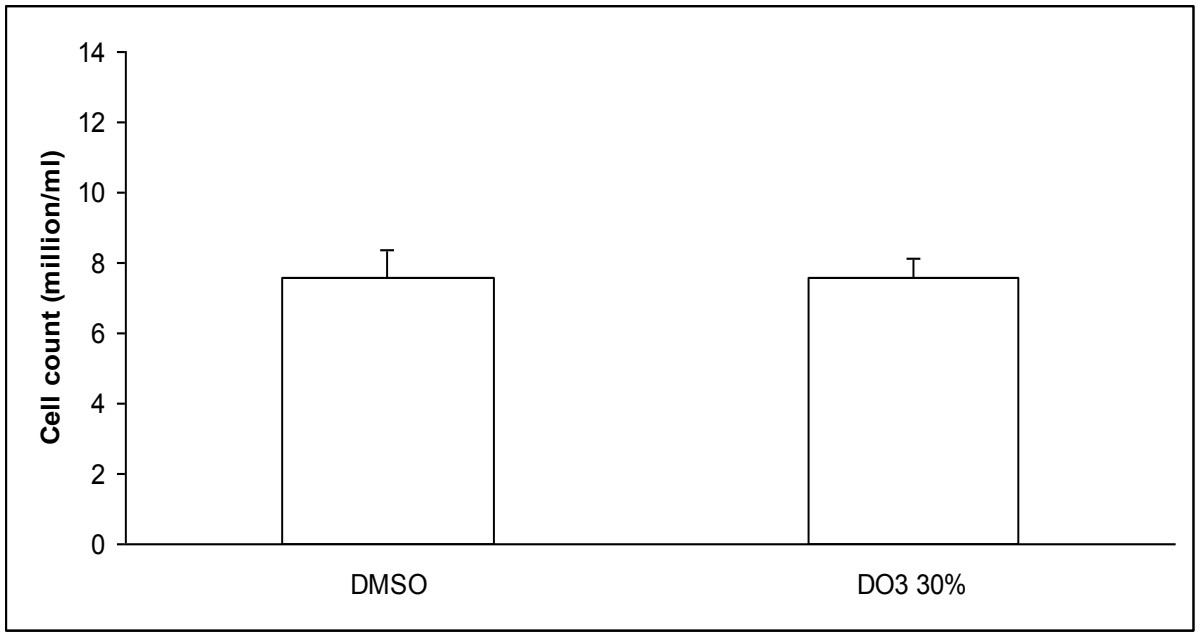


Fig. 3.1.1.7 Cell count (mean ± SD) values obtained from mice treated with Disperse Orange 3 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

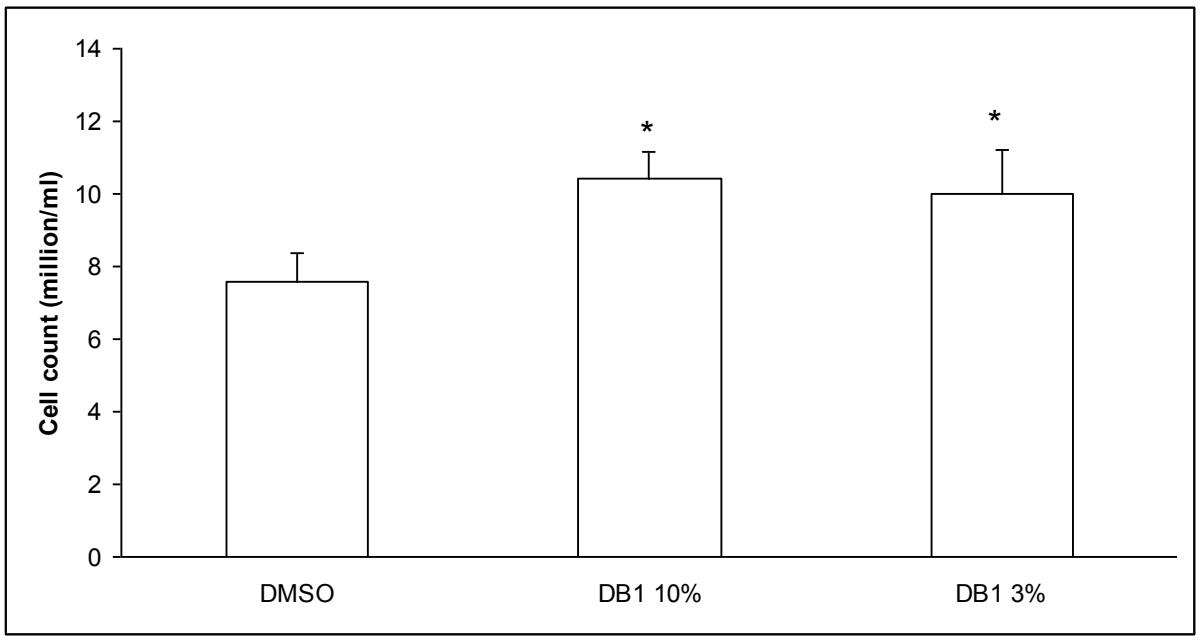


Fig. 3.1.1.8 Cell count (mean ± SD) values obtained from mice treated with Disperse Blue 1 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

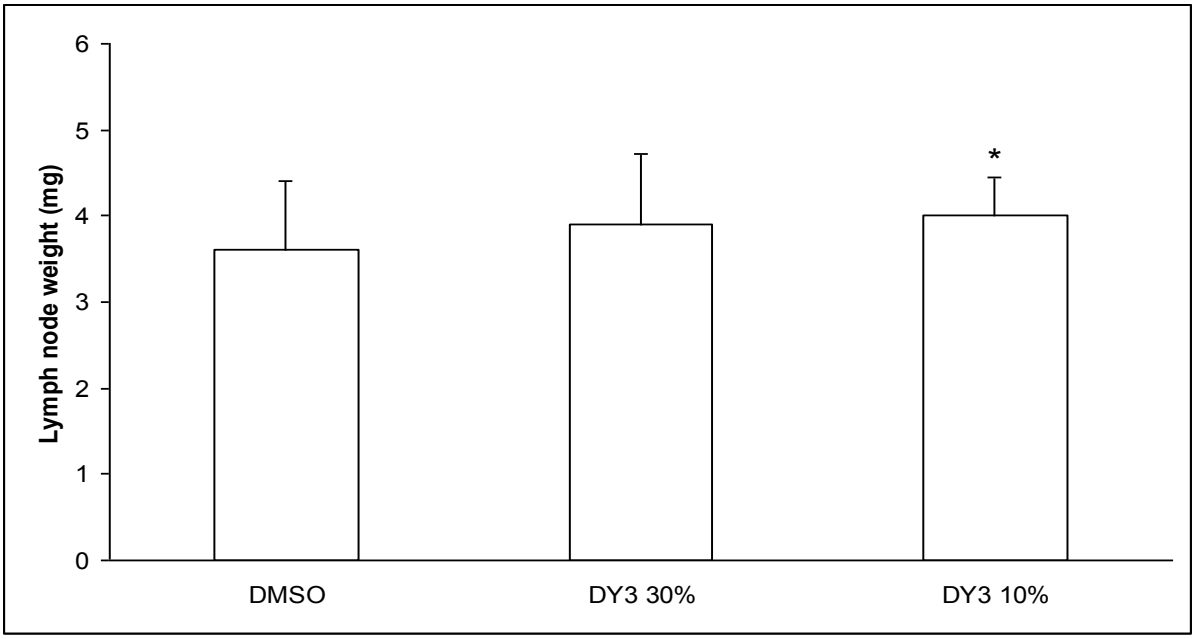


Fig. 3.1.2.1 Lymph node weight (mean ± SD) values obtained from mice treated with Disperse Yellow 3 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

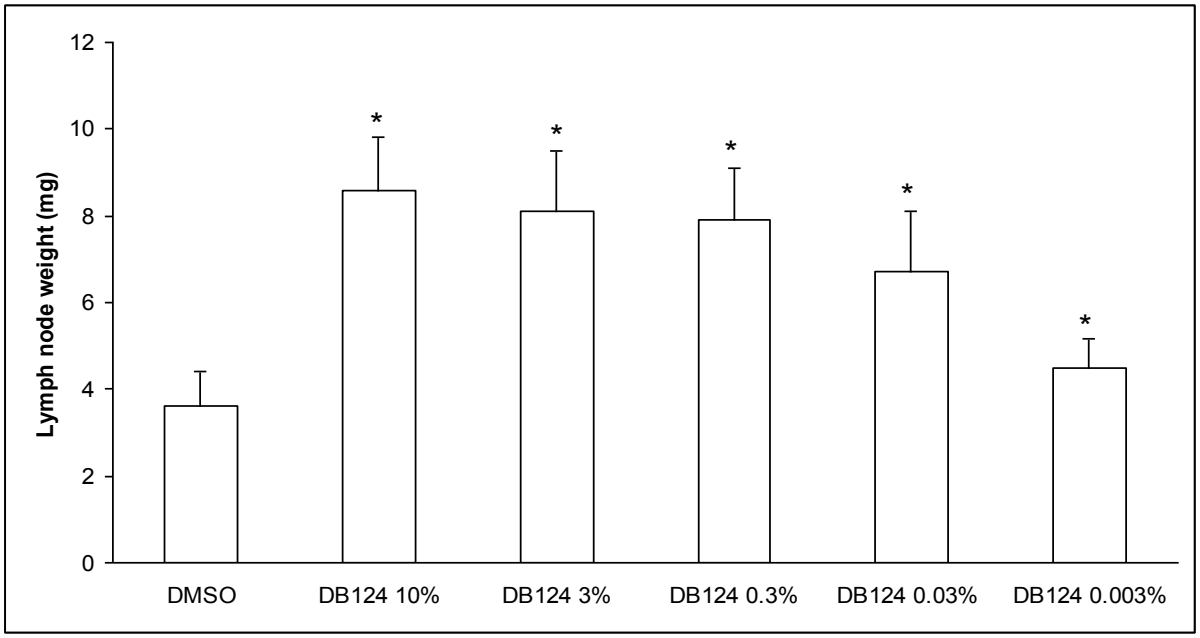


Fig. 3.1.2.2 Lymph node weight (mean ± SD) values obtained from mice treated with Disperse Blue 124 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

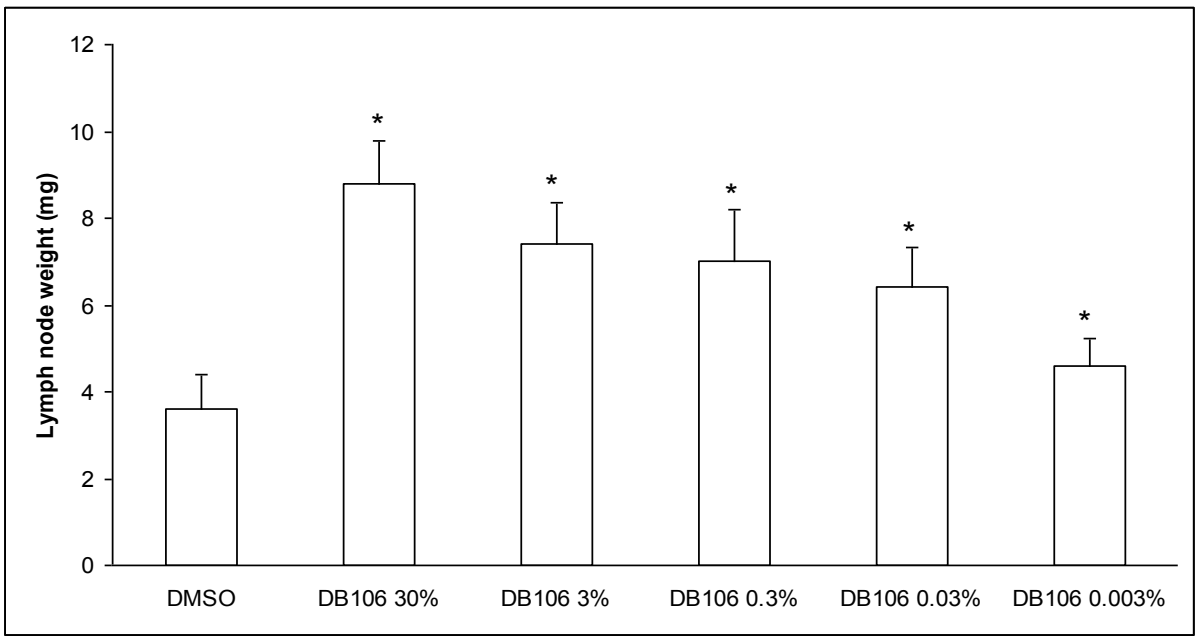


Fig. 3.1.2.3 Lymph node weight (mean ± SD) values obtained from mice treated with Disperse Blue 106 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

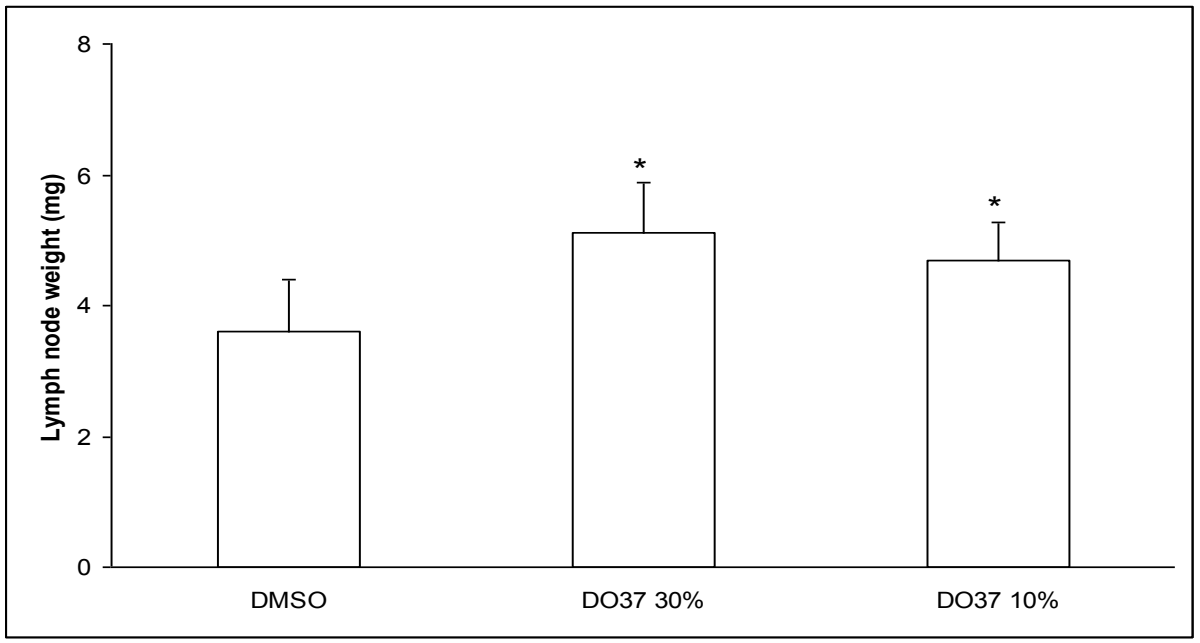


Fig. 3.1.2.4 Lymph node weight (mean ± SD) values obtained from mice treated with Disperse Orange 37 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

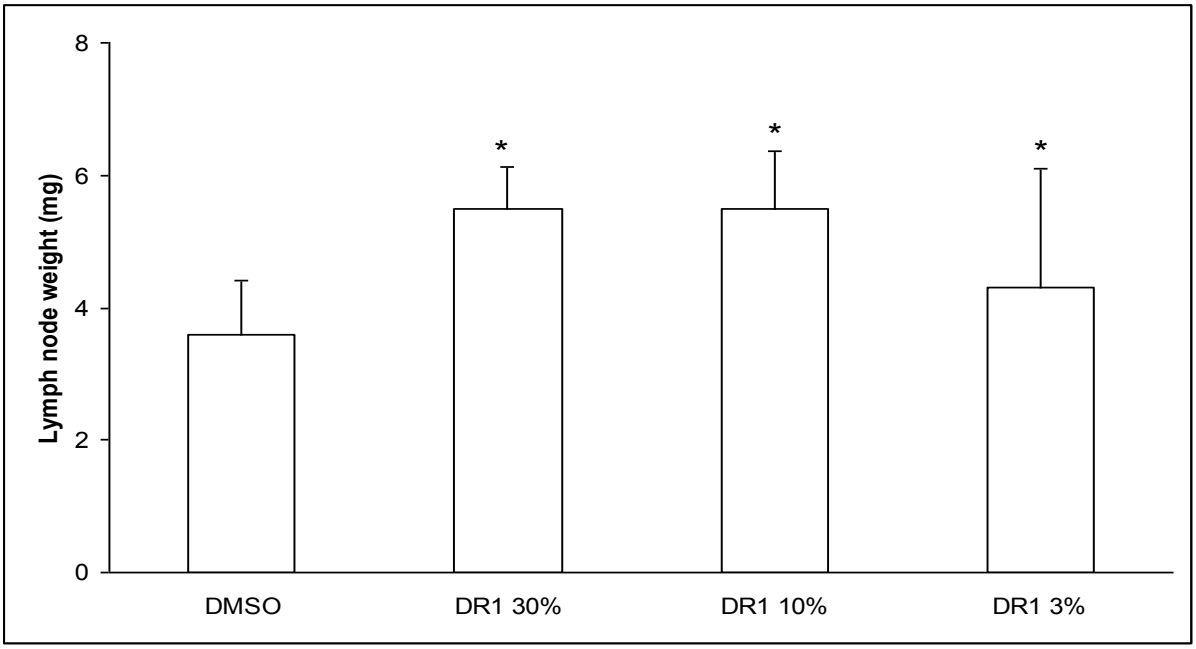


Fig. 3.1.2.5 Lymph node weight (mean ± SD) values obtained from mice treated with Disperse Red 1 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

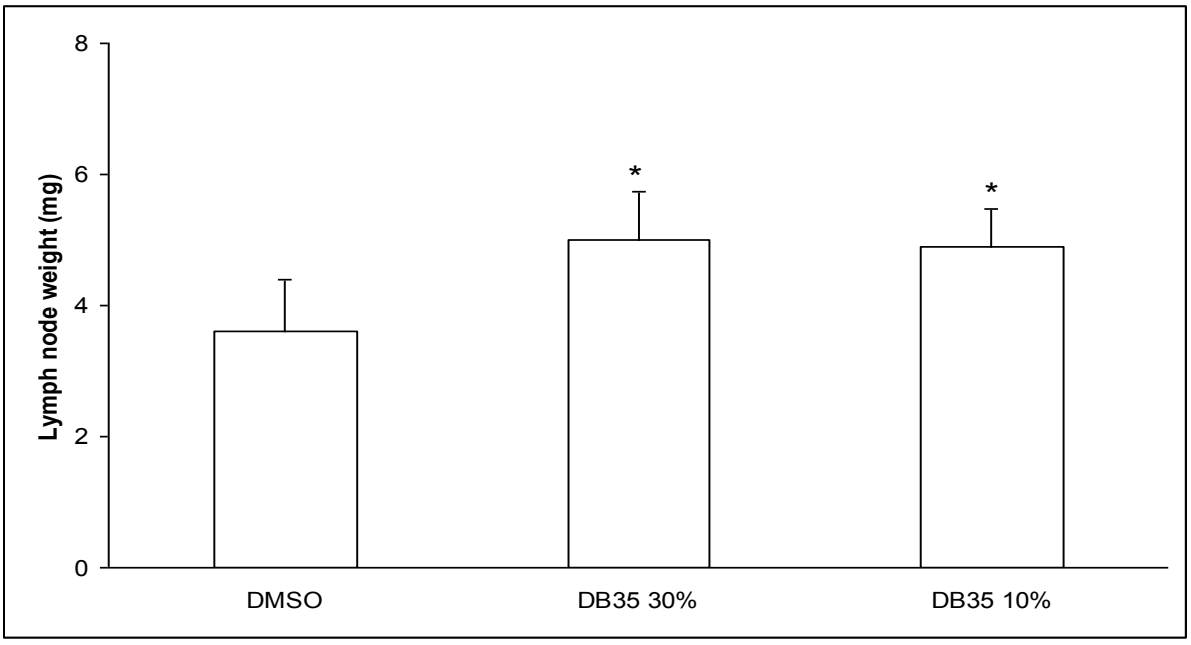


Fig. 3.1.2.6 Lymph node weight (mean ± SD) values obtained from mice treated with Disperse Blue 35 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

Application of Disperse blue 35 at 30 and 10% concentrations resulted in a significant amplification in lymph node weight in the treated mice by 39 and 36%, respectively, as compared to the DMSO treated control mice (Fig. 3.1.2.6).

No difference was observed in the lymph node weight of mice treated with Disperse orange 3 at 30% concentration, as compared to the vehicle treated mice (Fig. 3.1.2.7).

In the experiment involving application of Disperse blue 1 at 10 and 3% concentrations, significant enhancement in the lymph node weight was observed (33%) in the treated mice as compared to the vehicle treated control mice (Fig. 3.1.2.8).

3.1.3 Ear thickness measurement

Application of Disperse yellow 3 at 30 and 10% concentrations resulted in a significant increase in ear-thickness by 4% in the treated mice as compared to the vehicle control mice (Fig. 3.1.3.1).

In the study involving treatment with Disperse blue 124 at 10, 3, 0.3, 0.03 and 0.003% concentrations, there was observed significant increase in ear-thickness in the treated mice by 22, 26, 30, 4 and 4%, respectively, in comparison to the vehicle control mice (Fig. 3.1.3.2).

Treatment of mice with Disperse blue 106 at 30, 3, 0.3 and 0.03% concentrations resulted in significant increase in ear thickness by 26, 13, 17 and 9%, respectively, while no significant difference was noticed at 0.003% concentration in the treated animals as compared to the control animals (Fig. 3.1.3.3).

A significant increase of 4% in ear-thickness was observed in mice treated with Disperse orange 37 at 30% concentration, in comparison to the vehicle control mice. We did not find any difference of ear thickness in the mice treated with Disperse orange 37 at 10% concentration, as compared to the vehicle control mice group (Fig. 3.1.3.4).

In the study involving application of Disperse red 1 at 30, 10 and 3% concentrations, there was observed a significant increase (4%) in ear-thickness at 10% concentration, while no change was observed at 30 and 3% concentrations in the treated mice as compared to the vehicle control mice (Fig. 3.1.3.5).

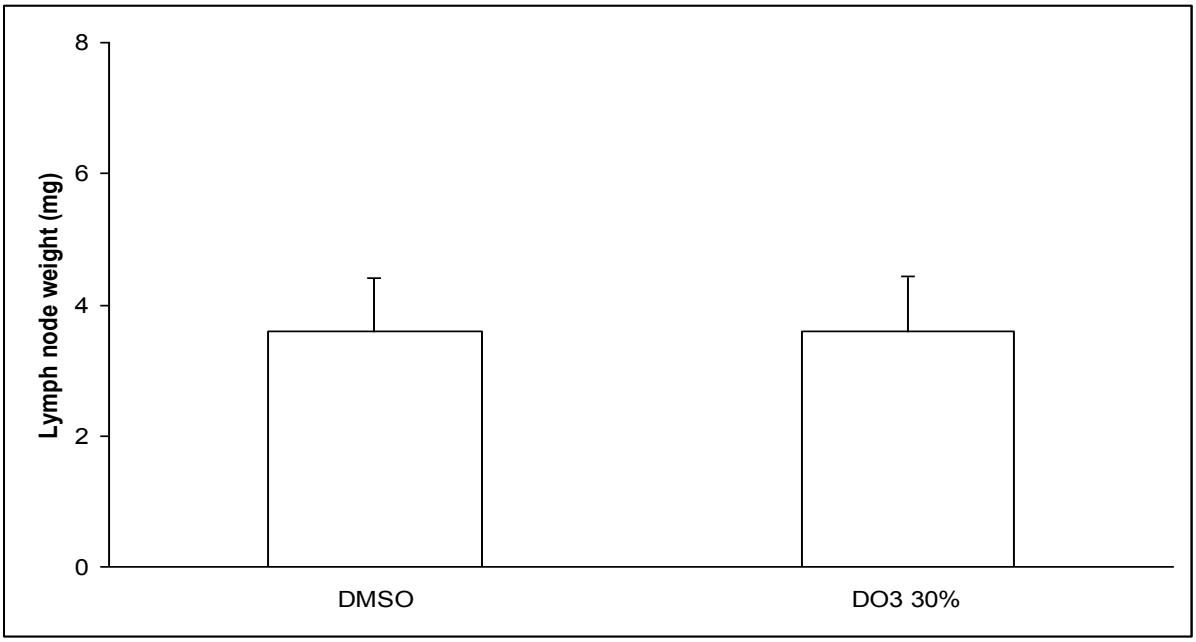


Fig. 3.1.2.7 Lymph node weight (mean ± SD) values obtained from mice treated with Disperse Red 1 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

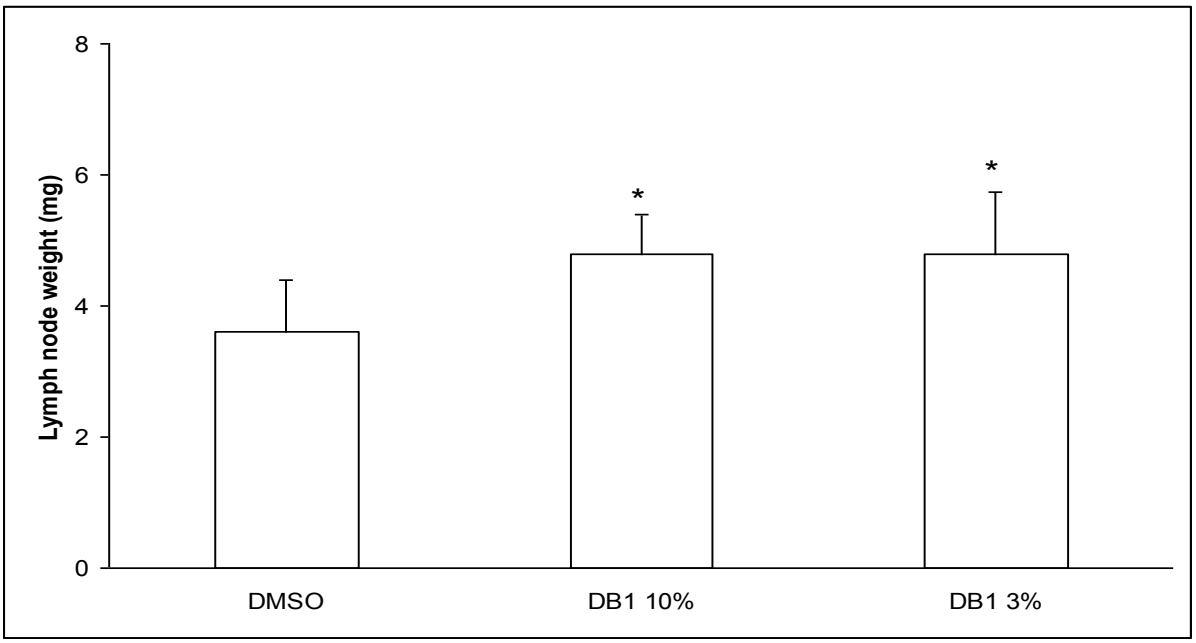


Fig. 3.1.2.8 Lymph node weight (mean ± SD) values obtained from mice treated with Disperse Blue 1 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

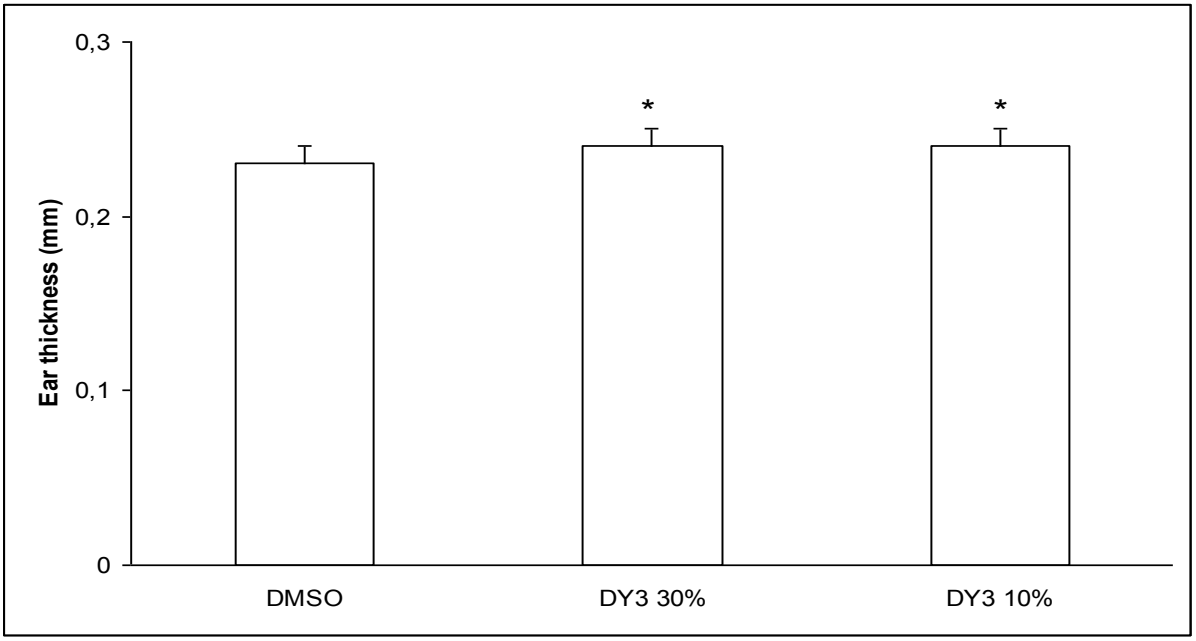


Fig. 3.1.3.1 Ear thickness (mean ± SD) values obtained from mice treated with Disperse Yellow 3 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

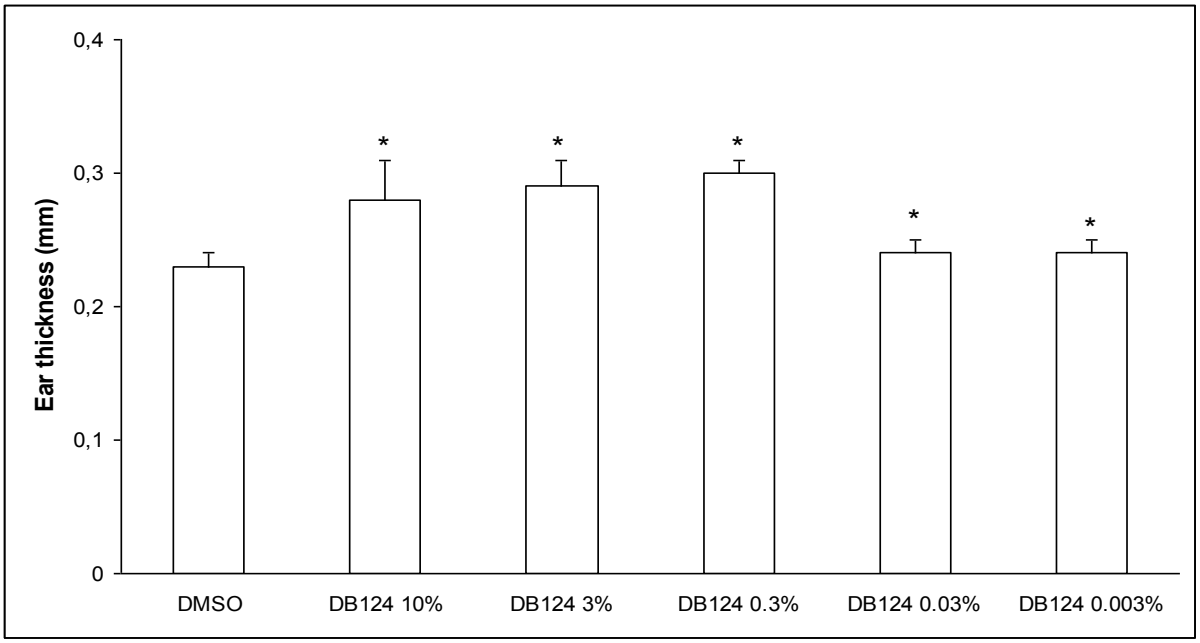


Fig. 3.1.3.2 Ear thickness (mean ± SD) values obtained from mice treated with Disperse Blue 124 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

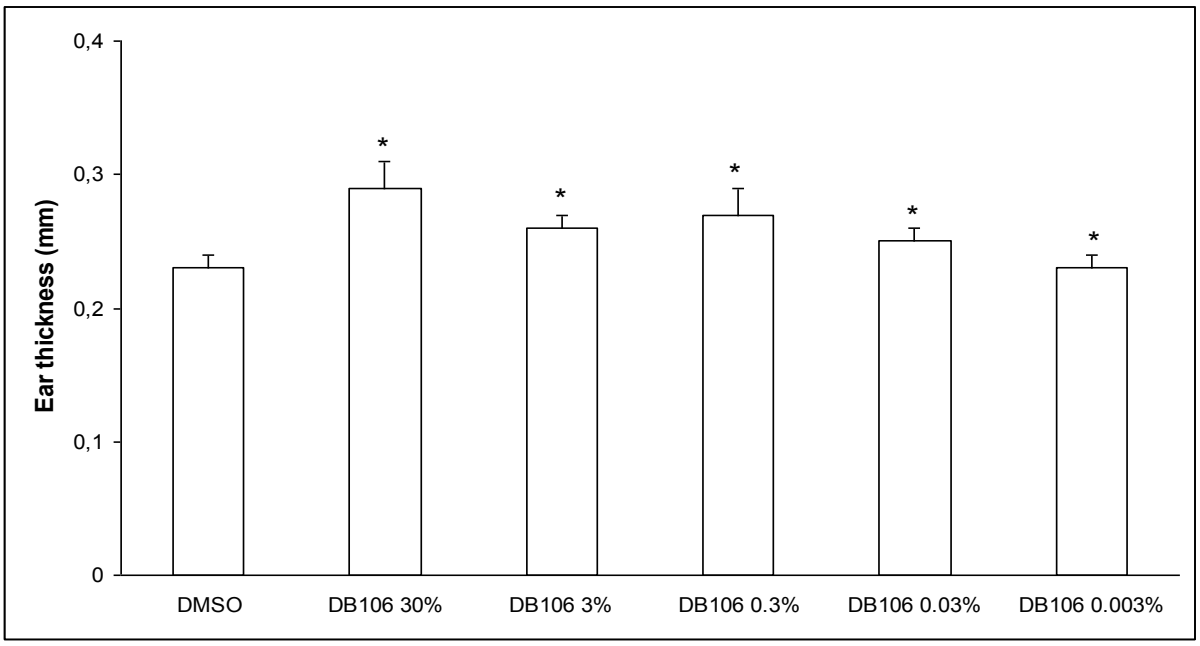


Fig. 3.1.3.3 Ear thickness (mean ± SD) values obtained from mice treated with Disperse Blue 106 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

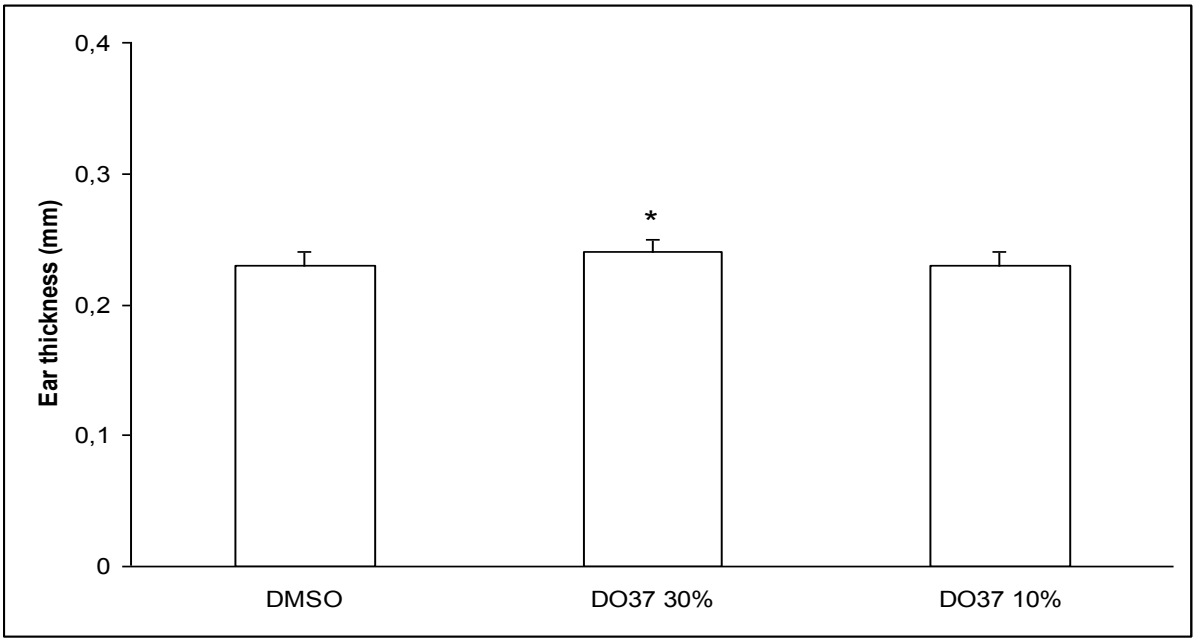


Fig. 3.1.3.4 Ear thickness (mean ± SD) values obtained from mice treated with Disperse Orange 37 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

In the study involving application of Disperse red 1 at 30, 10 and 3% concentrations, there was observed a significant increase (4%) in ear-thickness at 10% concentration, while no change was observed at 30 and 3% concentrations in the treated mice as compared to the vehicle control mice (Fig. 3.1.3.5).

Disperse blue 35 treatment at 30 and 10% concentrations resulted in a significant (9%) increase in ear-thickness in the treated mice as compared to the vehicle treated control mice (Fig. 3.1.3.6).

Following treatment with 30% solution of Disperse orange 3, no increase in ear-thickness was seen in the treated mice in comparison to the DMSO treated control mice (Fig. 3.1.3.7).

In the experiment with Disperse blue 1, there was found no change in ear-thickness at 10% concentration, while a significant increase of 9% was observed following 3% application in the treated mice as compared to the vehicle treated control mice (Fig. 3.1.3.8).

3.1.4 Ear-punch weight measurement

No relevant difference in the ear-punch weight was observed in the mice treated with a 30% solution of Disperse yellow 3, in comparison to the DMSO treated control mice. A significant modulation (+5%) in the ear-punch weight was seen in the mice treated with Disperse yellow 3 at 10% concentration, as compared to the DMSO treated control mice (Fig. 3.1.4.1).

Disperse blue 124 treatment at 10, 3, 0.3% concentrations showed significant amplification in ear-punch weight by 21, 22 and 28%, respectively, while at 0.03 and 0.003% concentrations, a non-significant increase of 4% was seen in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.4.2).

Application of Disperse blue 106 at 30, 3, 0.3 and 0.03% concentrations caused a significant enhancement in the ear-punch weight by 22, 15, 17 and 12%, respectively; while at 0.003% concentration a non-significant enhancement of 4% was noticed in the treated mice as compared to vehicle treated control mice (Fig. 3.1.4.3).

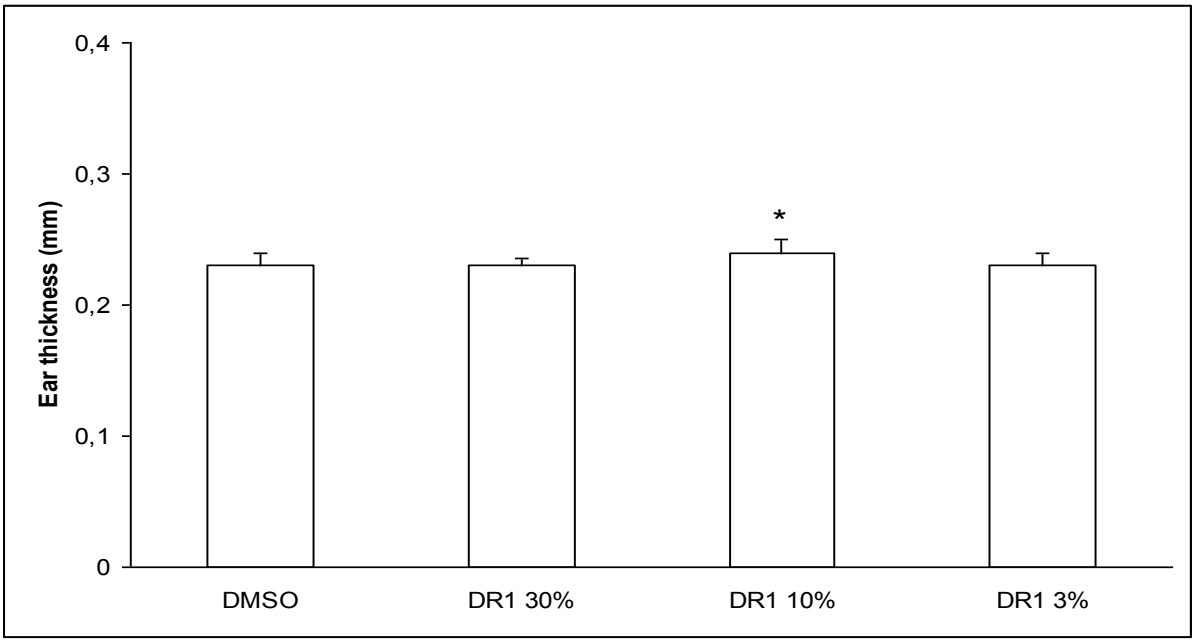


Fig. 3.1.3.5 Ear thickness (mean ± SD) values obtained from mice treated with Disperse Red 1 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

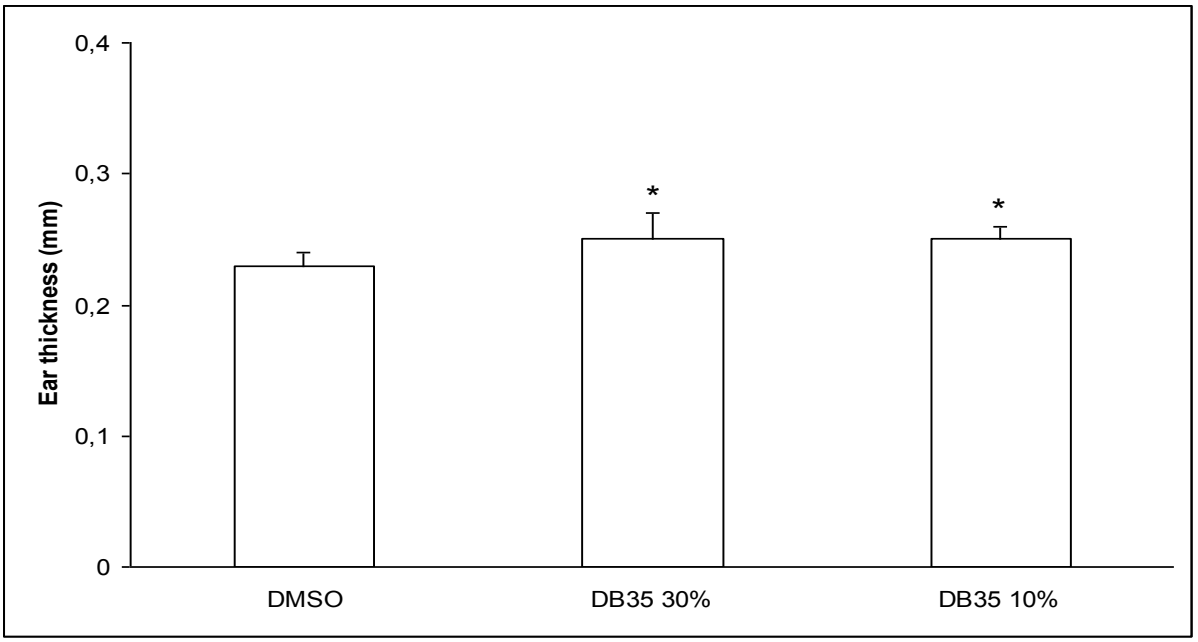


Fig. 3.1.3.6 Ear thickness (mean ± SD) values obtained from mice treated with Disperse Blue 35 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

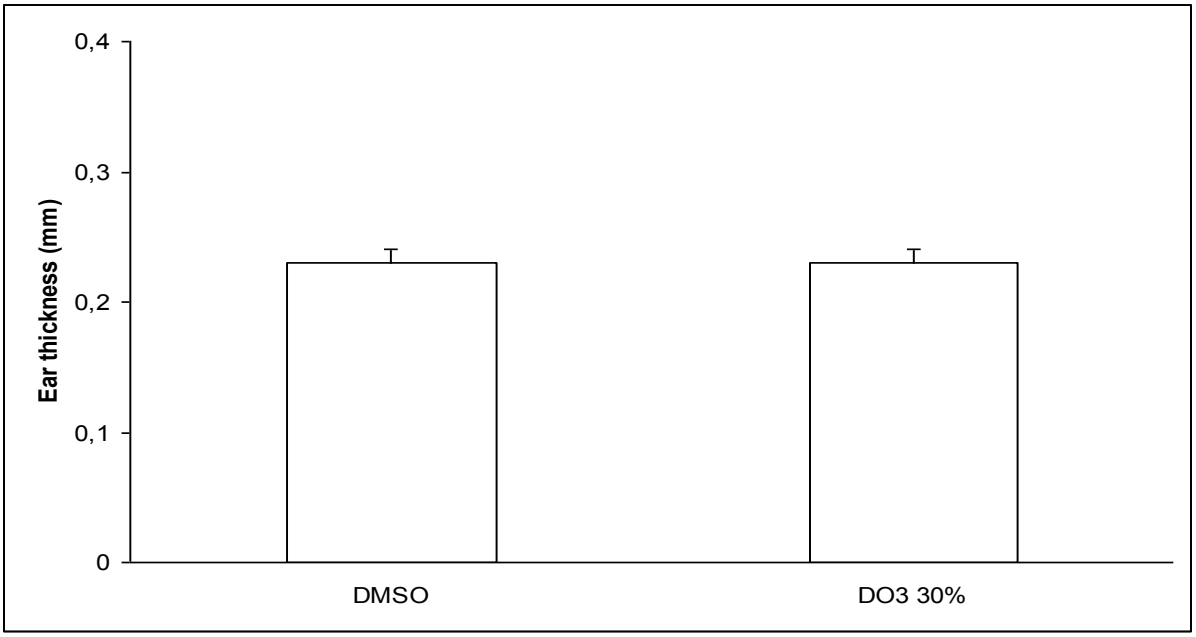


Fig. 3.1.3.7 Ear thickness (mean ± SD) values obtained from mice treated with Disperse Orange 3 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

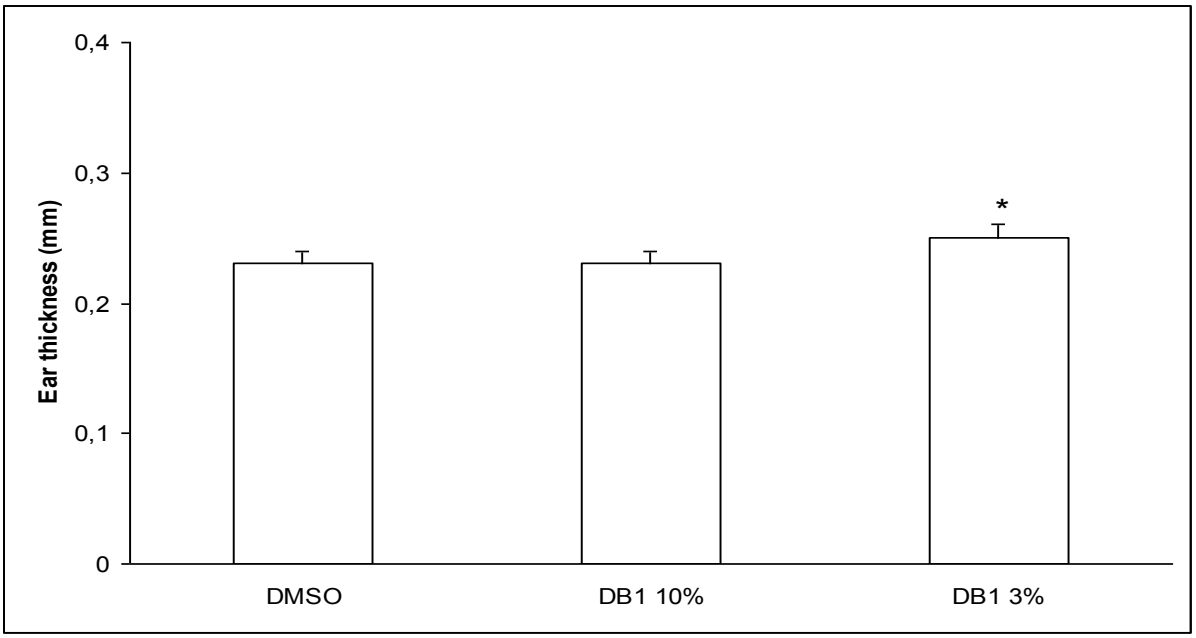


Fig. 3.1.3.8 Ear thickness (mean ± SD) values obtained from mice treated with Disperse Blue 1 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

In the experiment with Disperse orange 37 at 30% concentration, a non-significant increase of 4% in the ear-punch weight was seen between the treated and vehicle treated control animals (Fig. 3.1.4.4). At 10% concentration, no relevant difference was observed in the ear-punch weight.

In the study involving assessment of sensitisation potential of Disperse red 1 at 30, 10 and 3% concentrations, a significant augmentation (4%) was found in the ear-punch weight at 10% concentration, while no significant difference was observed at 30 and 3% concentrations, in the treated mice as compared to the vehicle treated control mice (Fig. 3.1.4.5).

Treatment with 30 and 10% solutions of Disperse blue 35 enhanced the ear-punch weight significantly by 6 and 7%, respectively, in the treated animals in comparison to the DMSO treated control animals (Fig. 3.1.4.6).

No increase in ear-punch weight was observed following treatment of mice with Disperse orange 3 at 30% concentration, in comparison to the control animals (Fig. 3.1.4.7).

Treatment of mice with Disperse blue 1 at 10% concentration showed no relevant difference in ear-punch weight, while treatment with 3% solution caused significant increase of 6% in the ear-punch weight of treated animals as compared to the control animals (Fig. 3.1.4.8).

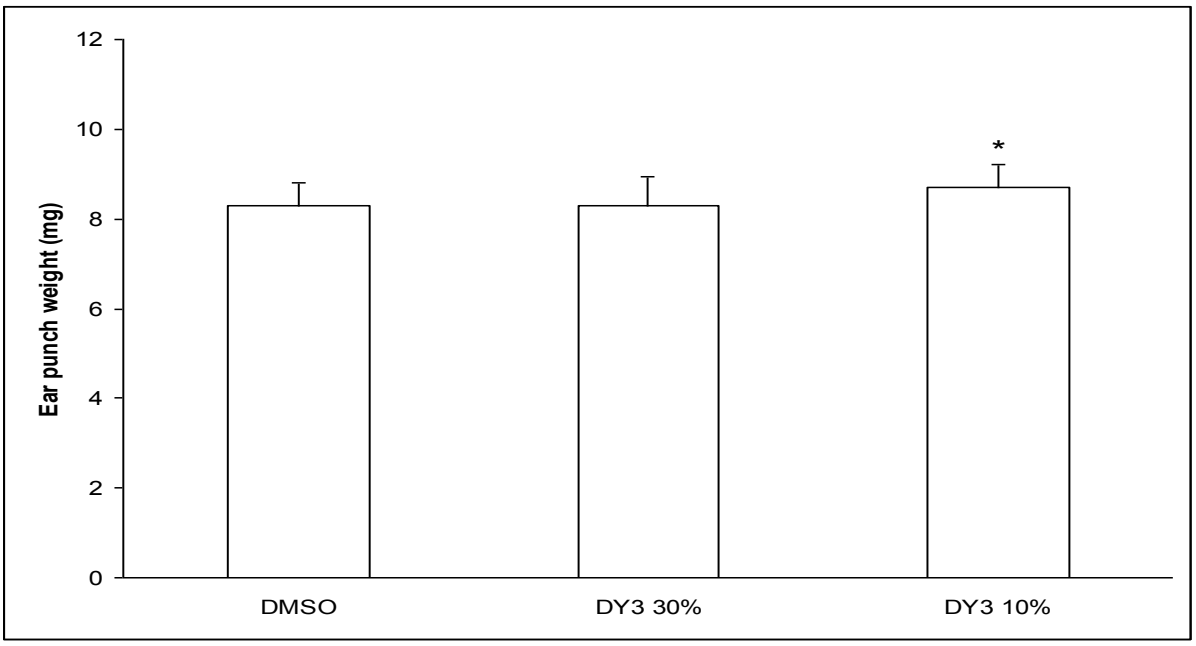


Fig. 3.1.4.1 Ear-punch weight (mean ± SD) values obtained from mice treated with Disperse Yellow 3 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

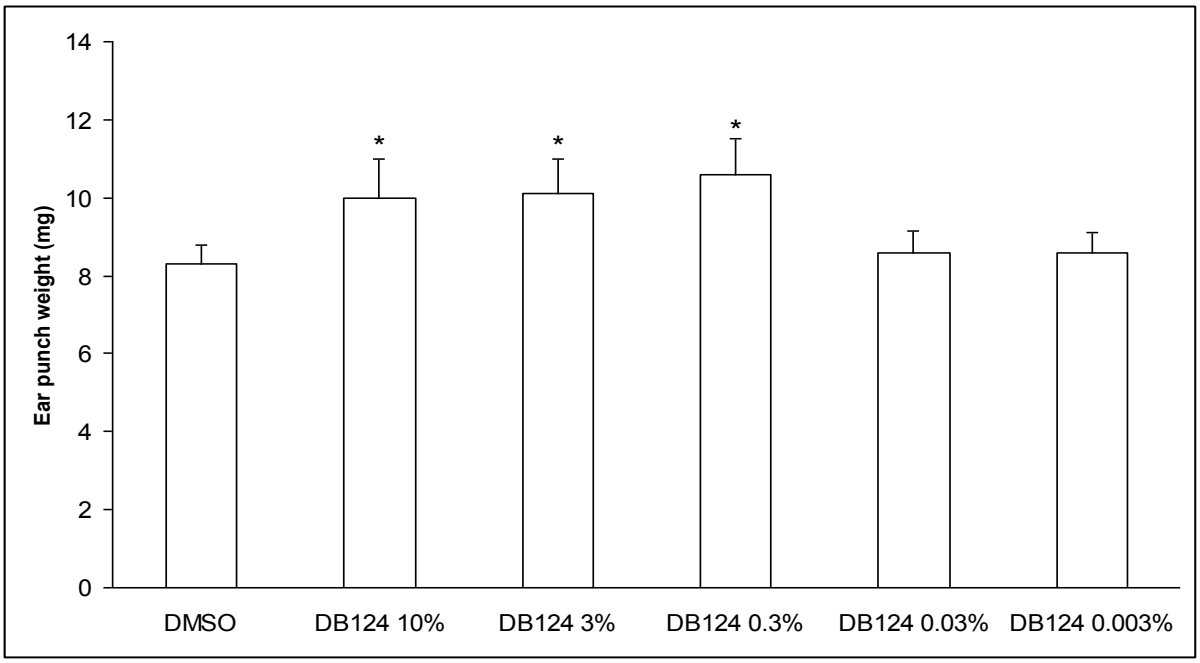


Fig. 3.1.4.2 Ear-punch weight (mean ± SD) values obtained from mice treated with Disperse Blue 124 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

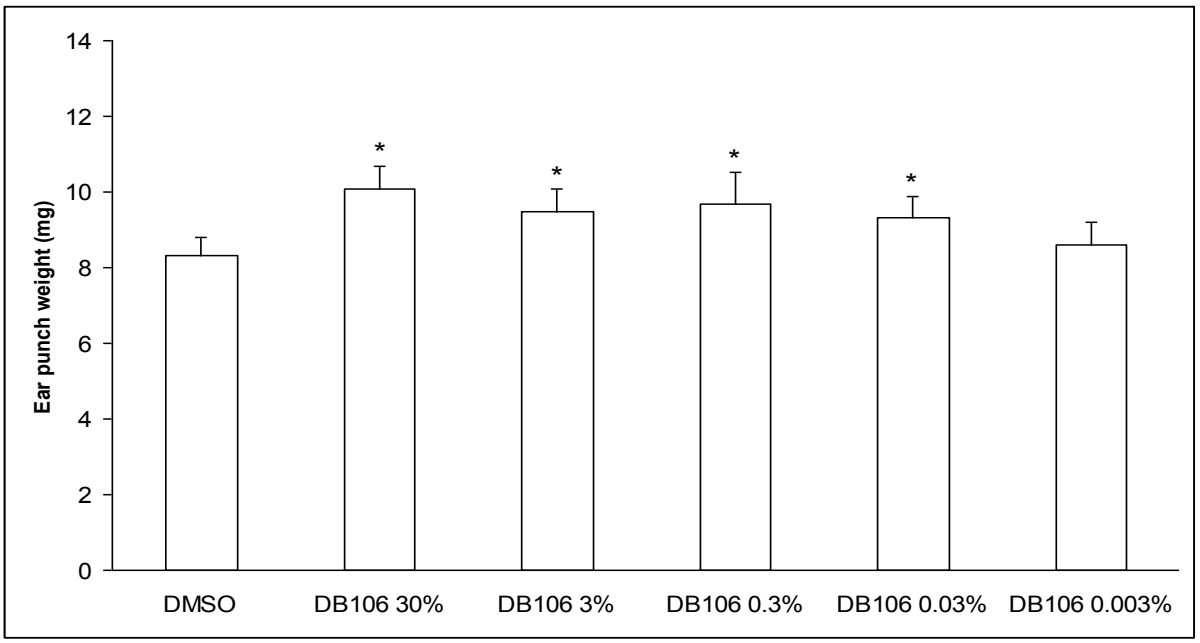


Fig. 3.1.4.3 Ear-punch weight (mean ± SD) values obtained from mice treated with Disperse Blue 106 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

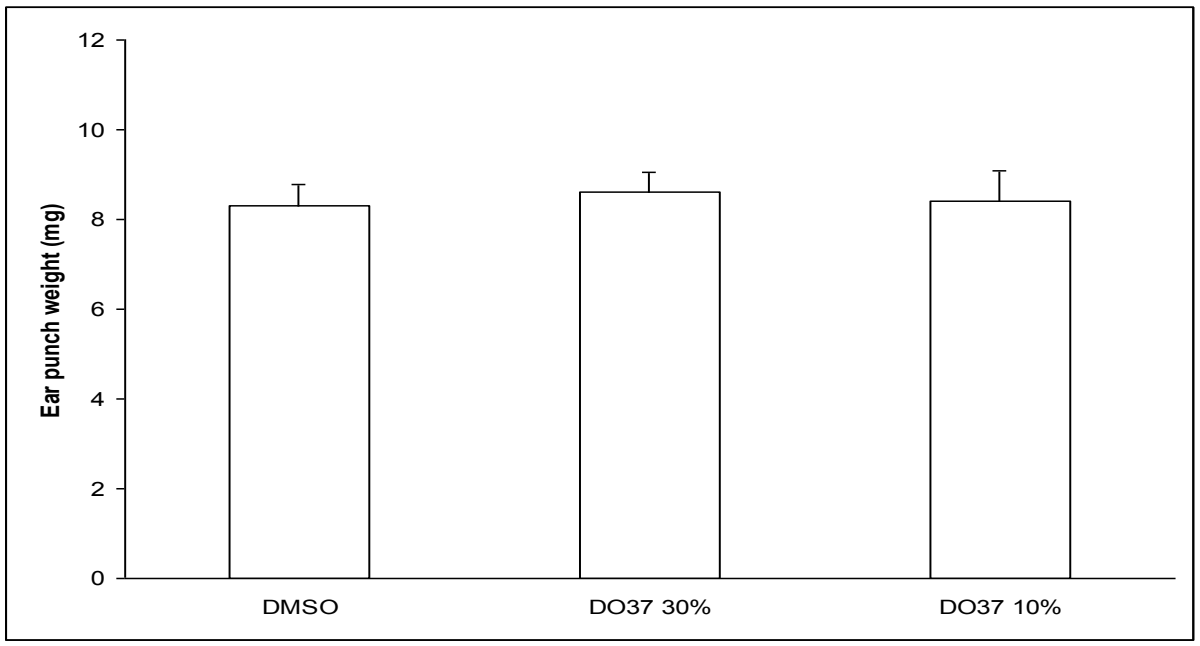


Fig. 3.1.4.4 Ear-punch weight (mean ± SD) values obtained from mice treated with Disperse Orange 37 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

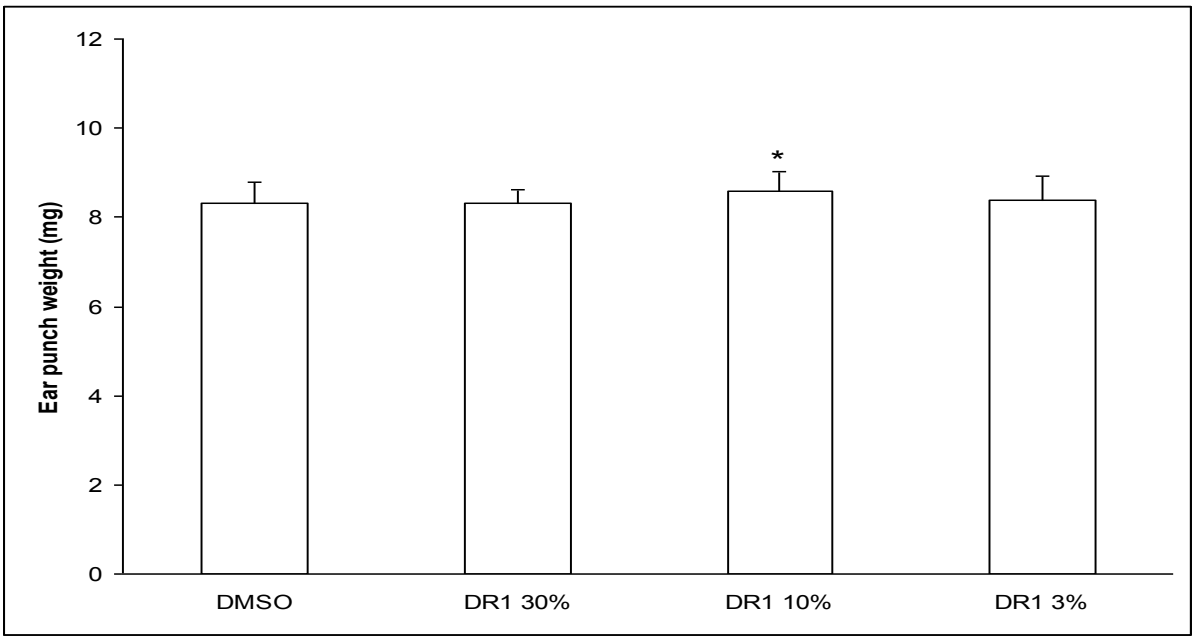


Fig. 3.1.4.5 Ear-punch weight (mean ± SD) values obtained from mice treated with Disperse Red 1 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

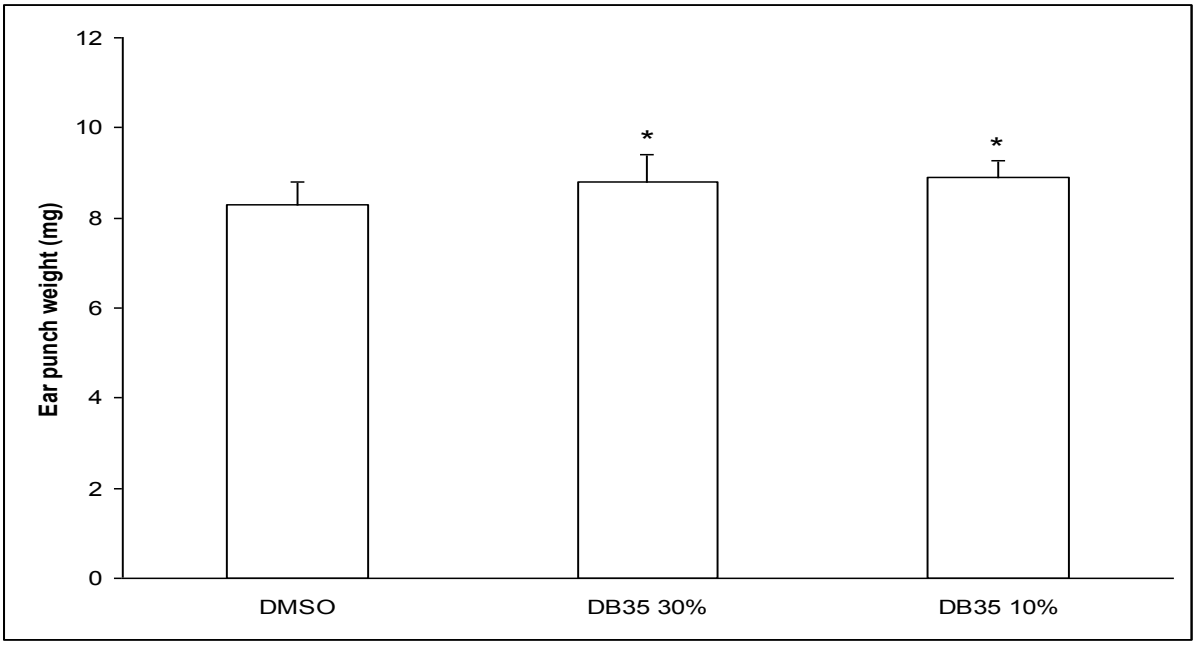


Fig. 3.1.4.6 Ear-punch weight (mean ± SD) values obtained from mice treated with Disperse Blue 35 according to the sensitisation-challenge protocol. *Significant increase at $p<0.05$ (t-test) between vehicle control (n=20) and treated animals (n=10).

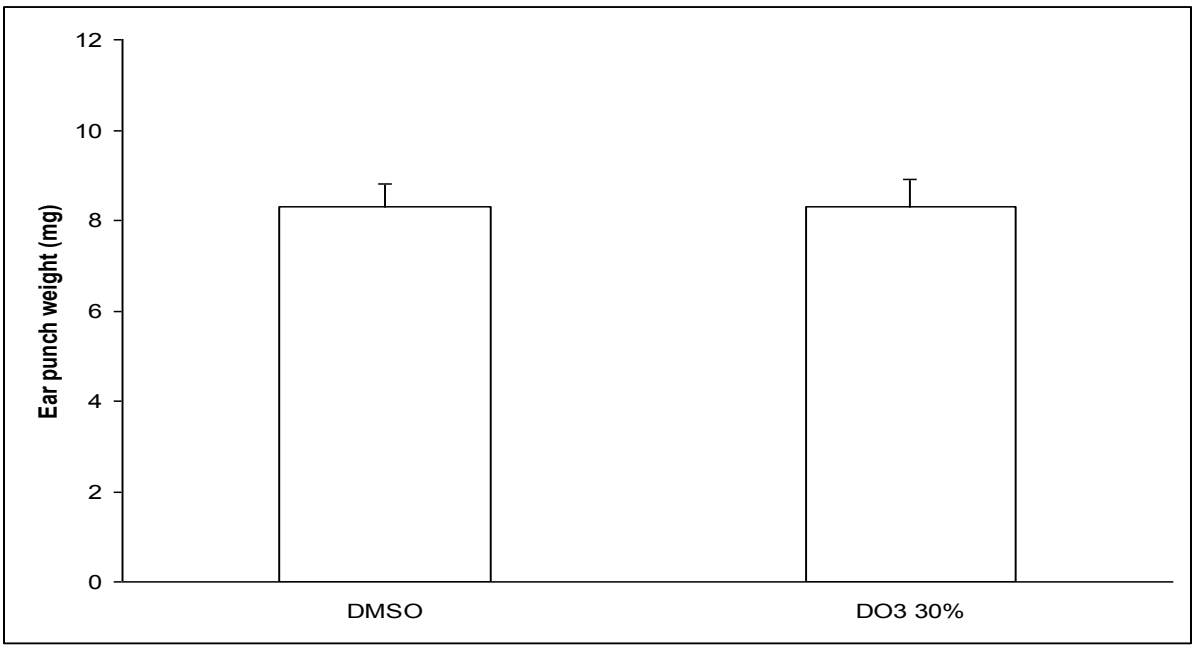


Fig. 3.1.4.7 Ear-punch weight (mean ± SD) values obtained from mice treated with Disperse Orange 3 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

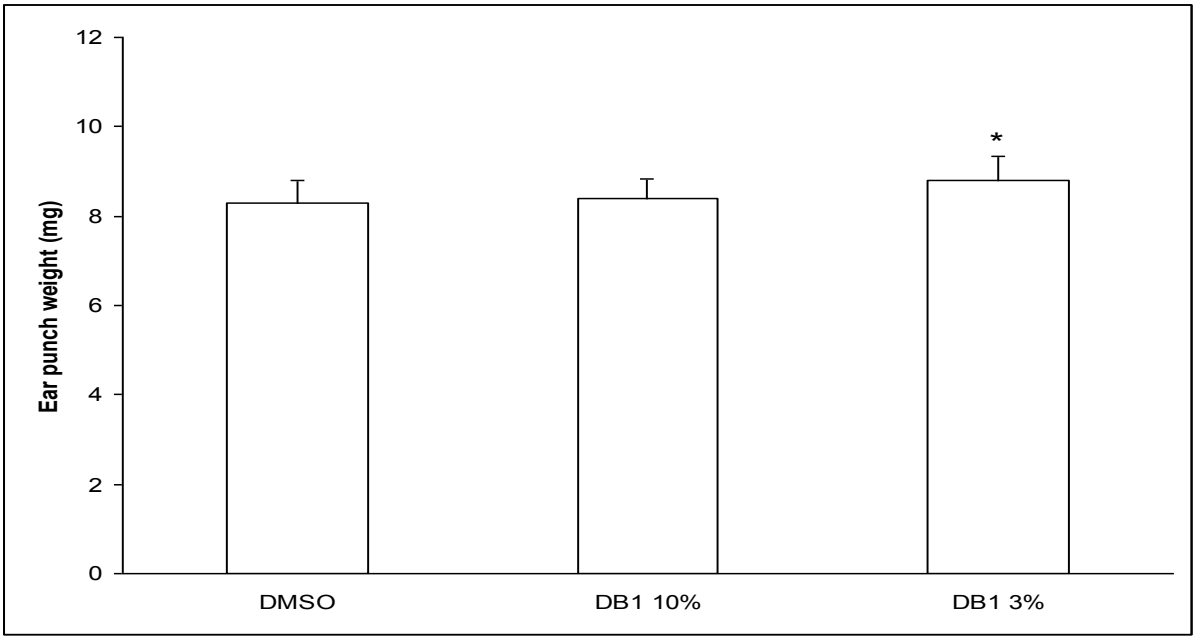
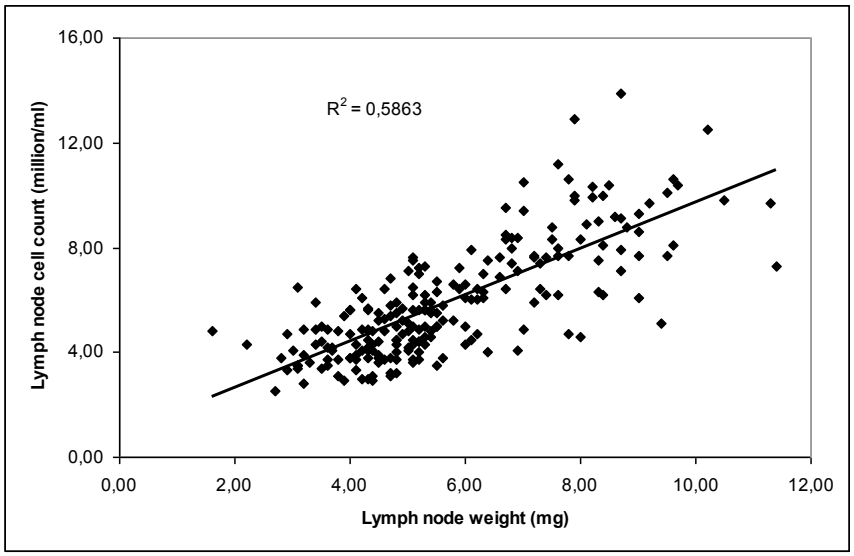


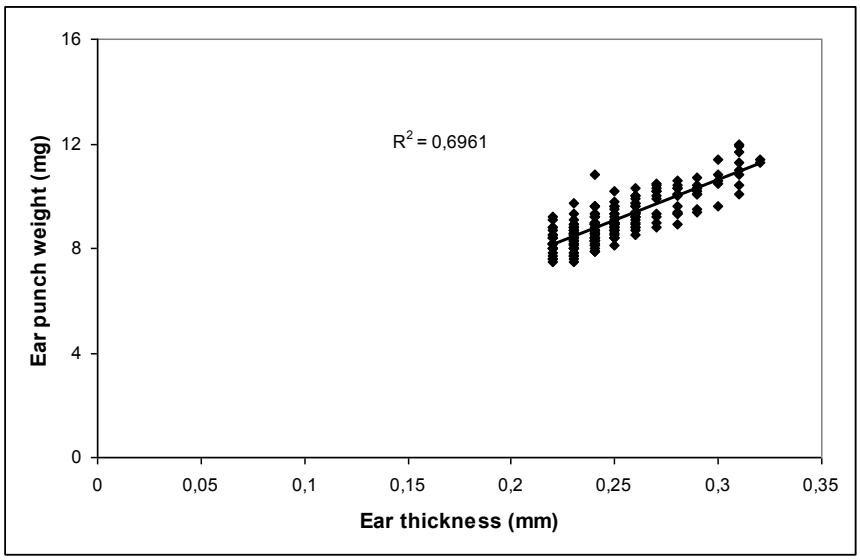
Fig. 3.1.4.8 Ear-punch weight (mean ± SD) values obtained from mice treated with Disperse Blue 1 according to the sensitisation-challenge protocol. *Significant increase at p<0.05 (t-test) between vehicle control (n=20) and treated animals (n=10).

The correlation between cell count and lymph node weight (Fig. 3.1.4.9 A), ear-thickness and ear punch weight (Fig. 3.1.4.9 B) for the textile dyes was determined by plotting a linear regression trend line fitted with Excel.

Fig. 3.1.4.10, Fig. 3.1.4.11, Fig. 3.1.4.12 and Fig. 3.1.4.13 represent the collective values for the lymph node cell count, lymph node weight, ear-thickness and ear-punch weight, respectively, for all the textile dyes tested in the biphasic local lymph node assay. Table 3.1.4.1 represents the numerical values for the lymph node and ear parameters.



(A)



(B)

Fig. 3.1.4.9 Correlation between lymph node cell count and lymph node weight (A), ear-thickness and ear-punch weight (B), following treatment of mice with various textile dyes. Linear regression trend line is fitted with Excel, R^2 value is given along with.

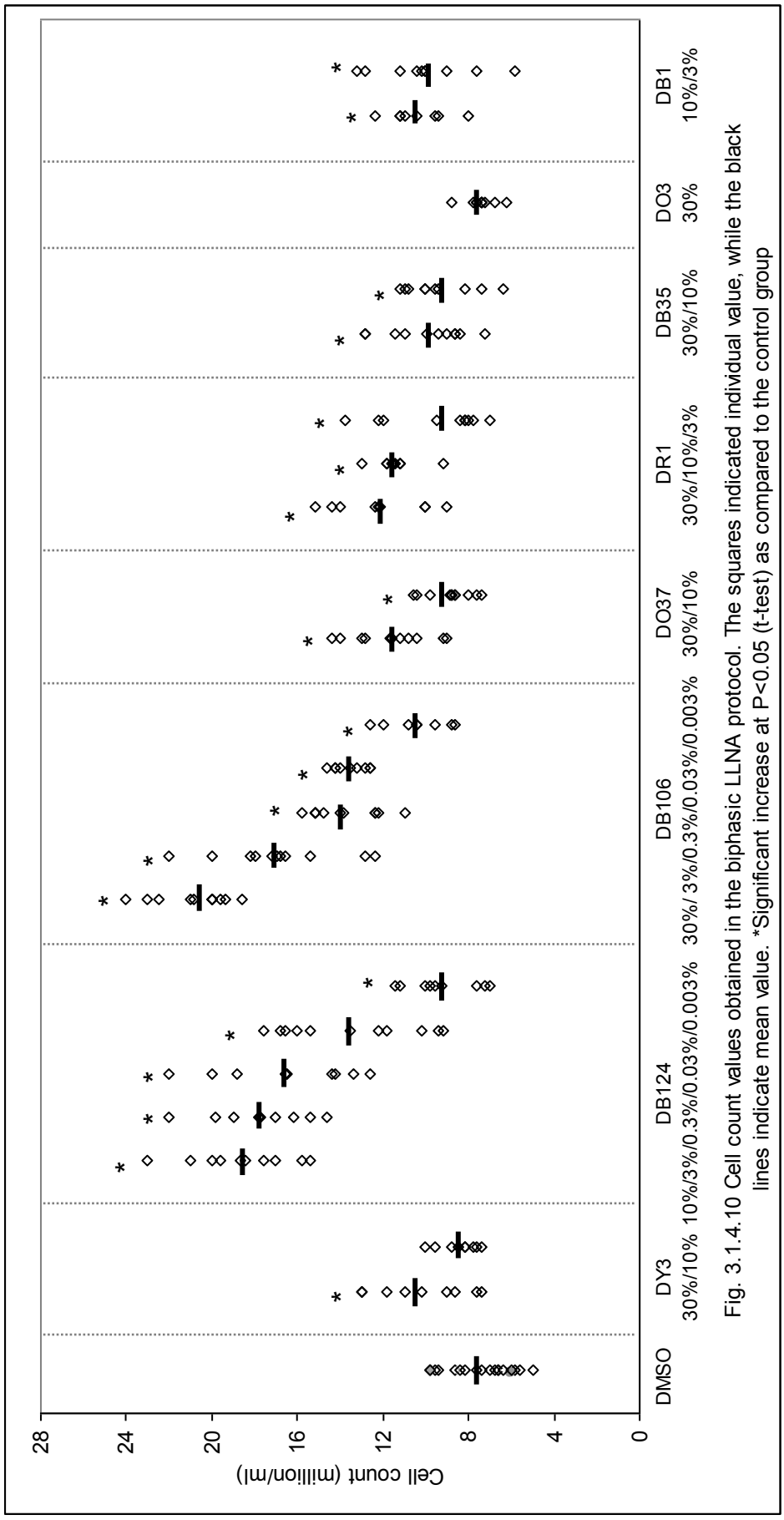


Fig. 3.1.4.10 Cell count values obtained in the biphasic LLNA protocol. The squares indicated individual value, while the black lines indicate mean value. *Significant increase at P<0.05 (t-test) as compared to the control group

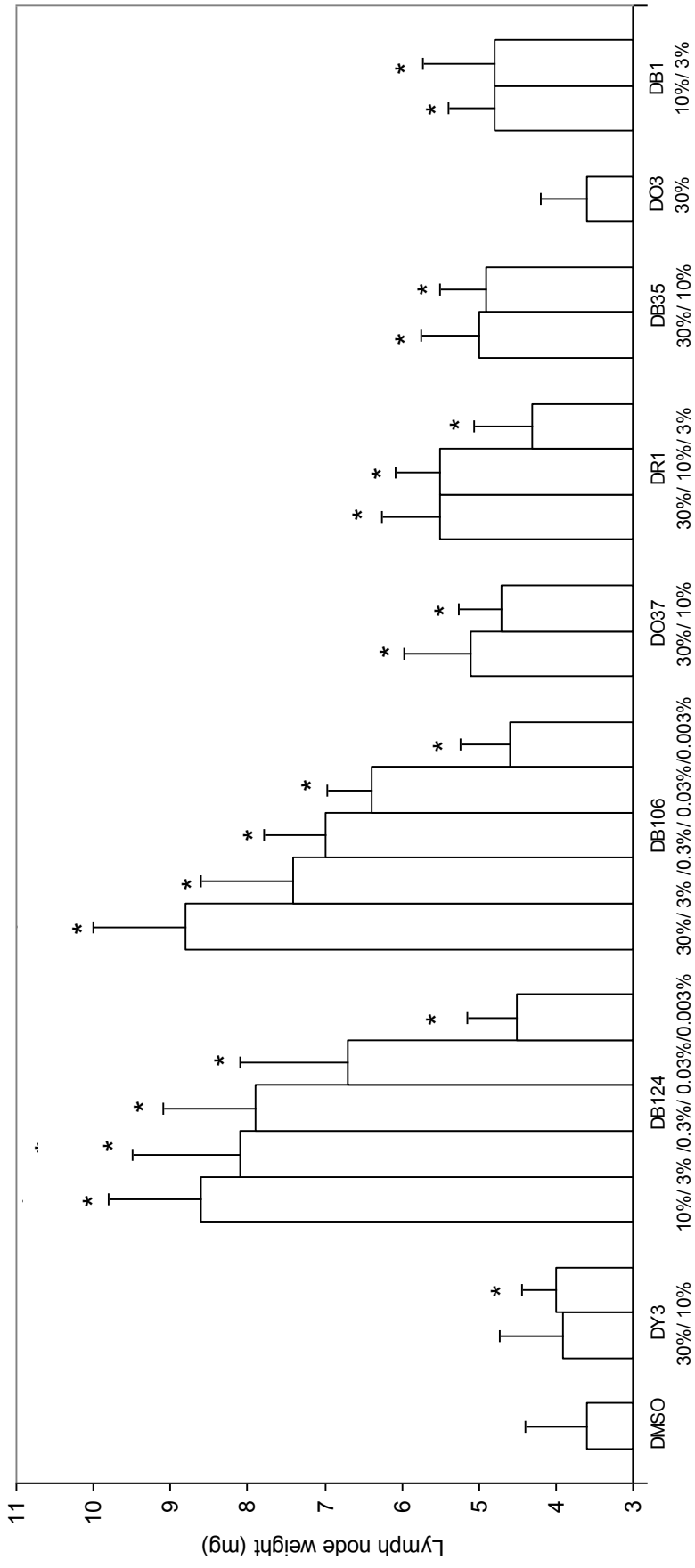


Fig. 3.1.4.11 Lymph node weight (mean ± SD) values obtained in the biphasic LLNA protocol. *Significant increase at $p<0.05$ (t-test). Minimum value on the y-axis is not 0, this is to give a better visual comparison of the results between various groups

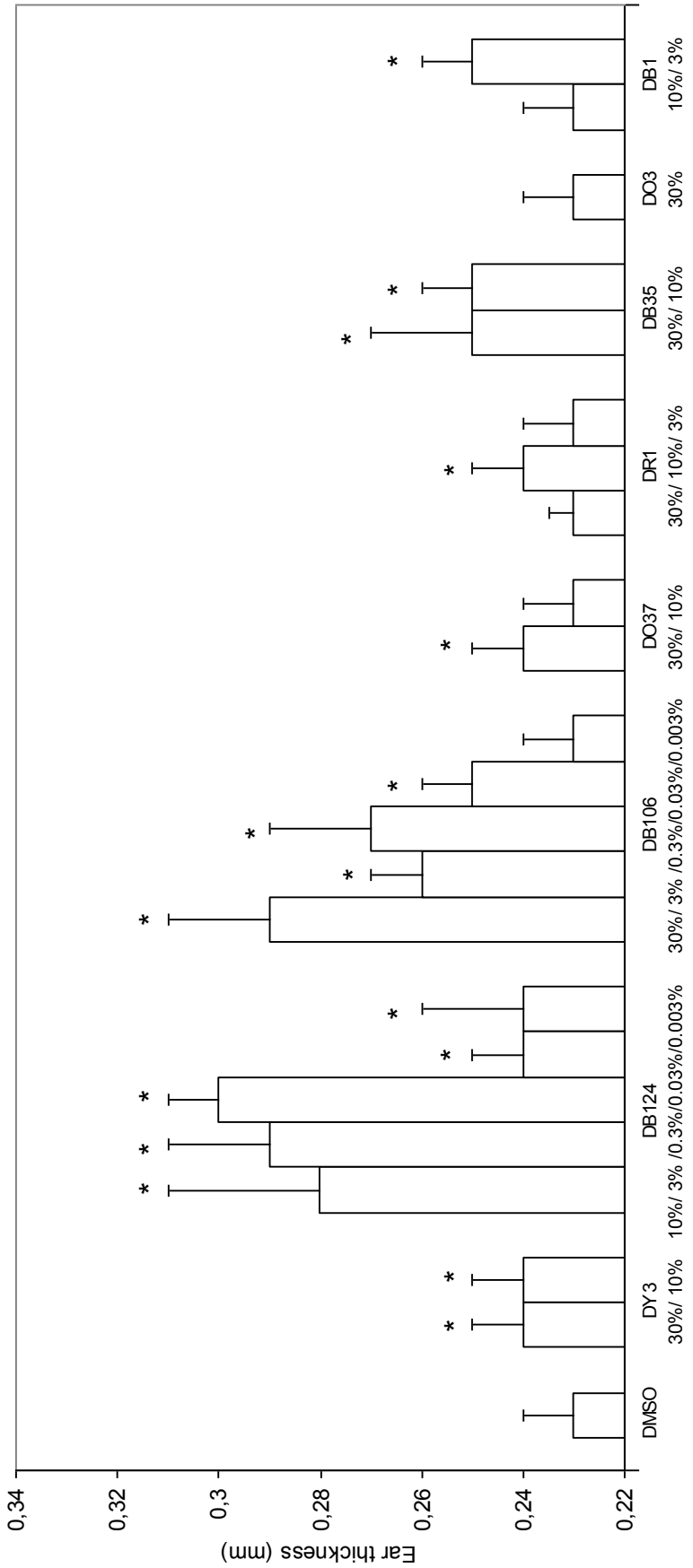


Fig. 3.1.4.12 Ear-thickness (mean ± SD) values obtained in the biphasic LLNA protocol. *Significant increase at $p<0.05$ (t-test). Minimum value on the y-axis is not 0, this is to give a better visual comparison of the results between various groups

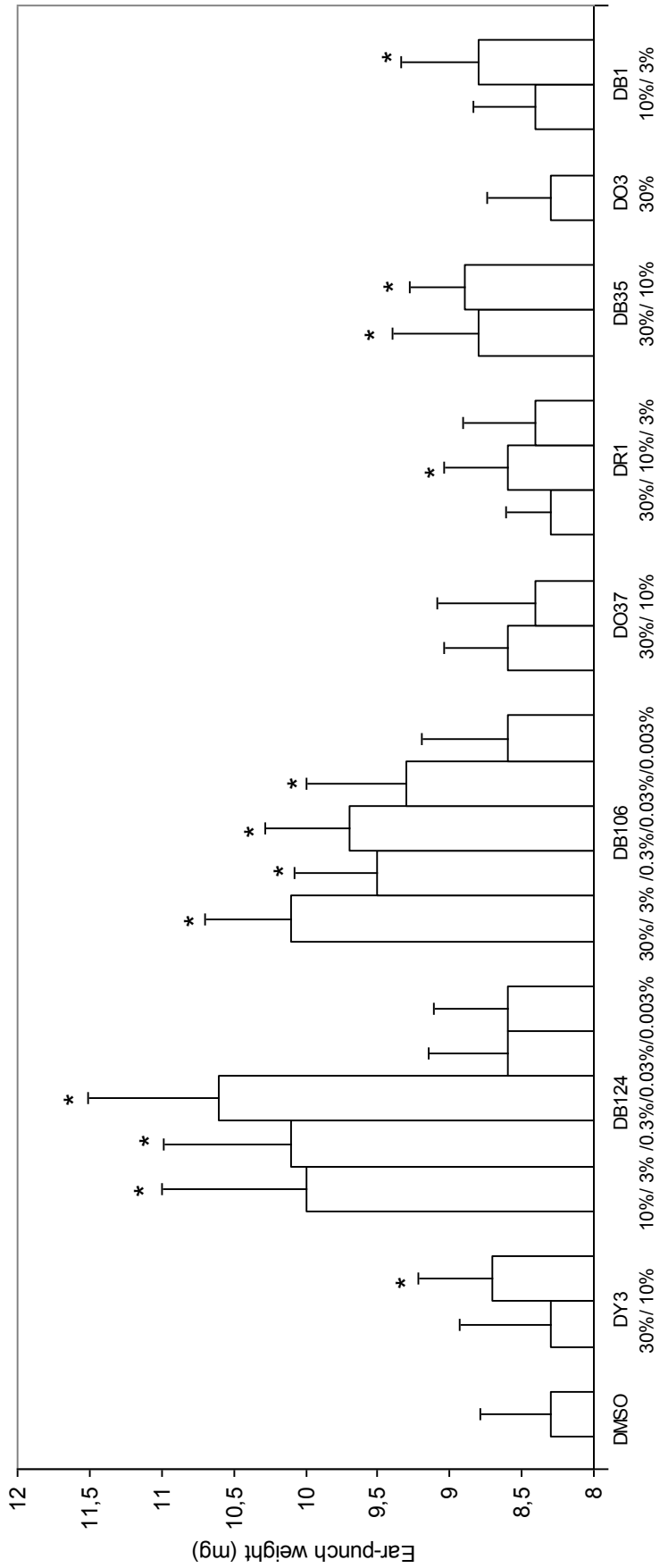


Fig. 3.1.4.13 Ear-punch weight (mean ± SD) values obtained in the biphasic LLNA protocol. *Significant increase at p<0.05 (t-test). Minimum value on the y-axis is not 0, this is to give a better visual comparison of the results between various groups.

Table 3.1.4.1 Values of the lymph node and ear parameters (Mean±SD) from mice treated with various disperse dyes according to the sensitisation-challenge protocol

		Cell count (million/ml)	Lymph node weight (mg)	Ear thickness (mm)	Ear-punch weight (mg)
1.	Vehicle control	7.6±0.77	3.6±0.8	0.23±0.01	8.3±0.49
2.	DY3 30%	10.2±1.2*	3.9±0.83	0.24±0.01*	8.3±0.63
	DY3 10%	8.4±0.47	4.0±0.45*	0.24±0.01*	8.7±0.52*
3.	DB124 10%	18.8±1.7*	8.6±1.2*	0.28±0.03*	10.0±1.0*
	DB124 3%	17.6±1.1*	8.1±1.4*	0.29±0.02*	10.1±0.88*
	DB124 0.3%	16.4±1.7*	7.9±1.2*	0.30±0.01*	10.6±0.91*
	DB124 0.03%	13.6±1.6*	6.7±1.4*	0.24±0.01*	8.6±0.55
	DB124 0.003%	9.2±0.87*	4.5±0.65*	0.24±0.01*	8.6±0.51
4.	DB106 30%	20.8±1.7*	8.8±1.0*	0.29±0.02*	10.1±0.6*
	DB106 3%	17.0±1.5*	7.4±0.96*	0.26±0.01*	9.5±0.58*
	DB106 0.3%	13.8±0.97*	7.0±1.2*	0.27±0.02*	9.7±0.84*
	DB106 0.03%	13.6±0.41*	6.4±0.92*	0.25±0.01*	9.3±0.58*
	DB106 0.003%	10.4±0.85*	4.6±0.63*	0.23±0.01	8.6±0.6
5.	DO37 30%	11.6±1.1*	5.1±0.78*	0.24±0.01*	8.6±0.44
	DO37 10%	8.8±0.55*	4.7±0.57*	0.23±0.01	8.4±0.69
6.	DR1 30%	12.2±1.2*	5.5±0.63*	0.23±0.005	8.3±0.31
	DR1 10%	11.4±0.96*	5.5±0.87*	0.24±0.01*	8.6±0.44*
	DR1 3%	9.6±1.2*	4.3±1.8	0.23±0.01	8.4±0.51
7.	DB35 30%	10.0±1.0*	5.0±0.75*	0.25±0.02*	8.8±0.6*
	DB35 10%	9.4±0.85*	4.9±0.58*	0.25±0.01*	8.9±0.38*
8.	DO3 30%	7.6±0.54	3.6±0.83	0.23±0.01	8.3±0.6
9.	DB1 10%	10.4±0.74*	4.8±0.6*	0.23±0.01	8.4±0.44
	DB1 3%	10.0±1.2*	4.8±0.93*	0.25±0.01*	8.8±0.54*

Sample size was n=10 in all the treated groups, n=20 for the vehicle control group. DMSO was used as vehicle. DY3 Disperse yellow 3, DB124 Disperse blue 124, DB106 Disperse blue 106, DO37 Disperse orange 37, DR1 Disperse red 1, DB35 Disperse blue 35, DO3 Disperse orange 3, DB1 Disperse blue 1. *Indicates significant change at $p<0.05$ (t-test) between vehicle control and treated animals.

3.1.5 Flow cytometry analysis

3.1.5.1 CD4+ cell population

Application of Disperse yellow 3 at 30% concentration resulted in a non-significant decrease (8%) in the CD4+ cell population in the treated animals as compared to the DMSO treated control animals. At 10% concentration of Disperse yellow 3, a significant decrease (9%) in the CD4+ population was observed in the lymphocytes from treated animals as compared to the DMSO treated control animals (Fig. 3.1.5.1).

In the study following application of Disperse blue 124 at 10, 3, 0.3, 0.03 and 0.003% concentrations, a significant decrease of 15, 22, 17, 19 and 15% in the CD4+ surface marker was found in the cells from treated mice as compared to vehicle control mice (Fig. 3.1.5.1).

Treatment with Disperse blue 106 at 30, 3, 0.3, 0.03 and 0.003% concentrations resulted in a significant decrease of CD4+ lymphocytes by 17, 20, 19, 25 and 15%, respectively, in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.5.1).

Disperse orange 37 treatment at 30 and 10% concentrations showed a significant decrease in the CD4+ epitope by 33 and 13%, respectively, in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.5.1).

Evaluation of Disperse red 1 at 30 and 10% concentrations for its sensitising potential resulted in a significant decrease in CD4+ lymphocytes by 24 and 9%, respectively, while at 3% concentration a non-significant decrease (3%) was observed in the treated mice as compared to the DMSO treated control mice (Fig. 3.1.5.1).

Disperse blue 35 application at 30 and 10% concentrations resulted in a significant decrease in CD4+ cell surface marker by 24 and 16%, respectively, in the treated mice as compared to the vehicle control mice (Fig. 3.1.5.1).

A significant decrease of 8% in the CD4+ cell population was observed in the mice treated with 30% solution of Disperse orange 3, as compared to the DMSO treated mice (Fig. 3.1.5.1).

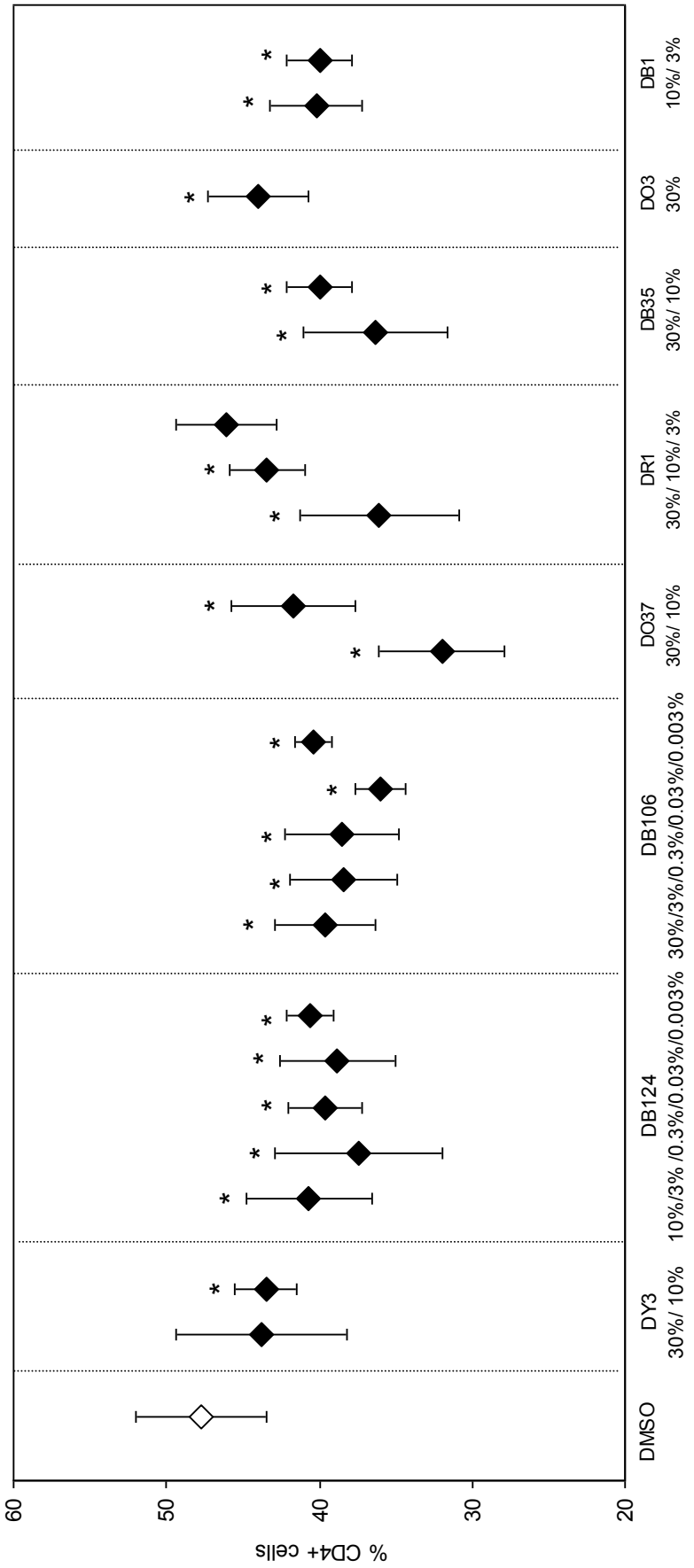


Fig. 3.1.5.1 CD4+ cell population (mean ± SD) from lymph nodes in the biphasic LLNA protocol. *Significant decrease at $p<0.05$ (t-test). Minimum value on the y-axis is not 0, to give a better visual comparison of the results between various groups.

Treating the mice with 10 and 3% solutions of Disperse blue 1 resulted in significant decrease in the CD4+ epitope marker by 16% in comparison to the vehicle treated control mice (Fig. 3.1.5.1).

3.1.5.2 CD8+ cell population

Disperse yellow 3 treatment at 30 and 10% concentrations did not cause any relevant modulation in CD8+ cells, in the treated animals as compared to the vehicle control animals (Fig. 3.1.5.2).

Application of 10, 3, 0.3, 0.03 and 0.003% solutions of Disperse blue 124 resulted in a significant decrease in the CD8+ cell surface marker by 12, 25, 10, 26 and 25%, respectively, in the treated animals as compared to the DMSO treated control animals (Fig. 3.1.5.2).

Treatment with 30, 3, 0.3, 0.03 and 0.003% concentrations of Disperse blue 106 showed significant decrease in CD8+ epitope on lymphocytes by 16, 20, 9, 22 and 27%, respectively, in the treated mice as compared to the vehicle treated mice (Fig. 3.1.5.2).

A significant decrease of 26% in the CD8+ cell population was observed following treatment of mice with Disperse orange 37 at 30% concentration, in comparison to mice treated with vehicle alone (Fig. 3.1.5.2). At 10% concentration, no relevant difference was observed in the CD8+ cell population in the treated and control mice.

In the study involving treatment with Disperse red 1 at 30% concentration, a significant decrease of 23% was observed in the CD8+ cells in the treated mice as compared to the vehicle treated mice (Fig. 3.1.5.2). No relevant difference was observed in the CD8+ cells following treatment with Disperse red 1 at 10 and 3% concentrations, in the treated mice as compared to the vehicle treated control mice.

Disperse blue 35 treatment at 30 and 10% concentrations showed a significant decrease of 20 and 7%, respectively, in the treated mice as compared to the vehicle treated control mice (Fig. 3.1.5.2).

A significant decrease of 8% in CD8+ cells was observed in the mice treated with Disperse orange 3 at 30% concentration as compared to the vehicle control mice (Fig. 3.1.5.2).

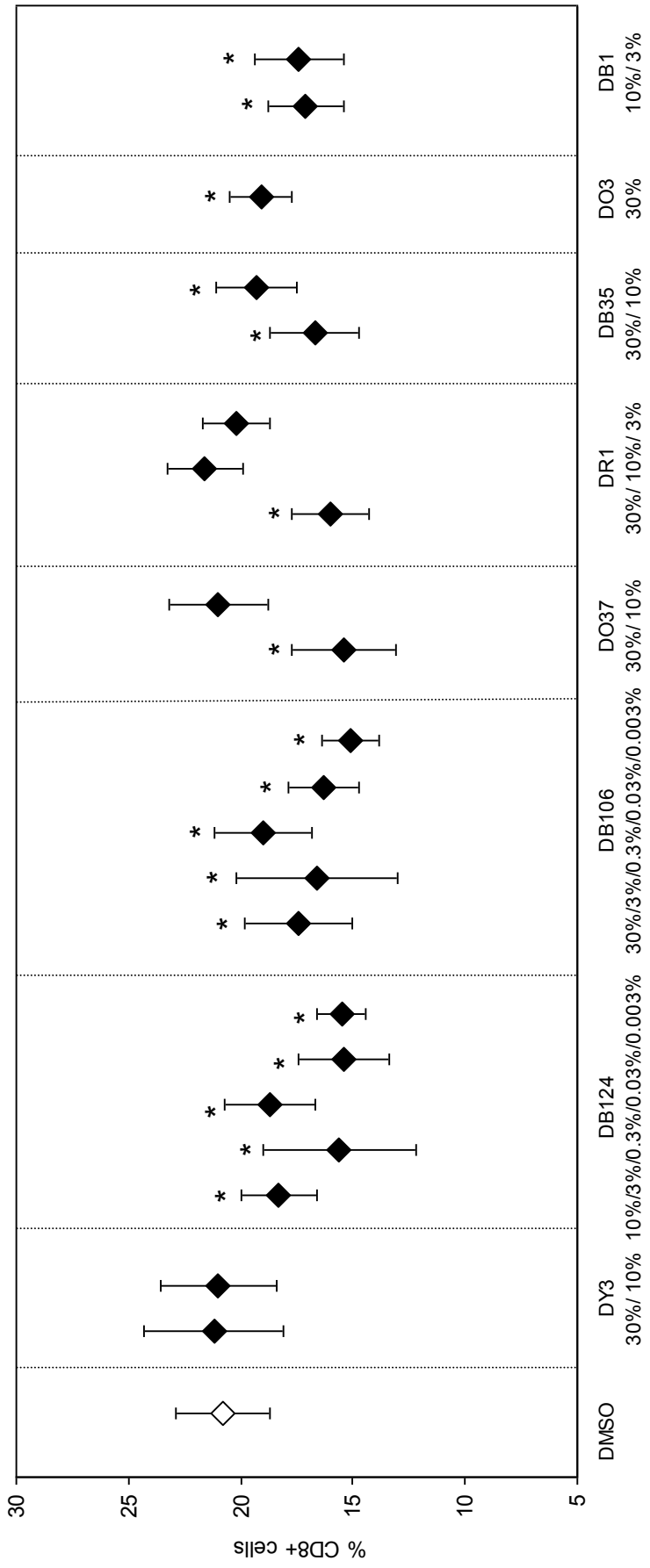


Fig. 3.1.5.2 CD8+ cell population (mean ± SD) from lymph nodes in the biphasic LLNA protocol. *Significant decrease at $p<0.05$ (t-test). Minimum value on the y-axis is not 0, to give a better visual comparison of the results between various groups

Study of the sensitising potential of Disperse blue 1 at 10 and 3% concentrations showed a significant decrease in the CD8+ lymphocytes by 18 and 16%, respectively, in the treated mice in comparison to the DMSO treated control mice (Fig. 3.1.5.2).

3.1.5.3 CD19+ cell population

Application of Disperse yellow 3 at 30 and 10% concentrations resulted in a significant increase in the CD19+ cells by 33 and 56%, respectively, in the treated mice as compared to the vehicle control mice (Fig. 3.1.5.3).

In the study involving treatment of mice with Disperse blue 124 at 10, 3, 0.3, 0.03 and 0.003% concentrations, a significant increase in CD19+ cell surface marker by 21, 104, 147, 98 and 78%, was observed in the treated mice in comparison to the DMSO treated control mice (Fig. 3.1.5.3).

Treatment with Disperse blue 106 at 30, 3, 0.3, 0.03 and 0.003% concentrations showed a significant increase of 33, 94, 81, 104 and 70%, respectively; in the CD19+ epitope measured in the treated animals in comparison to the vehicle control animals (Fig. 3.1.5.3).

A significant increase of 59 and 40% in CD19+ lymphocytes was observed in the mice treated with Disperse orange 37 at 30 and 10% concentrations, as compared to the vehicle treated control animals (Fig. 3.1.5.3).

Study of the sensitising potential of Disperse red 1 at 30, 10 and 3% concentrations showed a significant increase in CD19+ cell population by 68, 32 and 29%, respectively, in the treated mice as compared to the vehicle control mice (Fig. 3.1.5.3).

Disperse blue 35 treatment at 30 and 10% concentrations caused a significant increase of 98 and 72%, respectively, in CD19+ cells in the treated animals in comparison to the DMSO treated control animals (Fig. 3.1.5.3).

A significant increase of 35% was observed in the CD19+ cells in the mice treated with Disperse orange 3 at 30% concentration, in comparison to the vehicle treated control animals (Fig. 3.1.5.3).

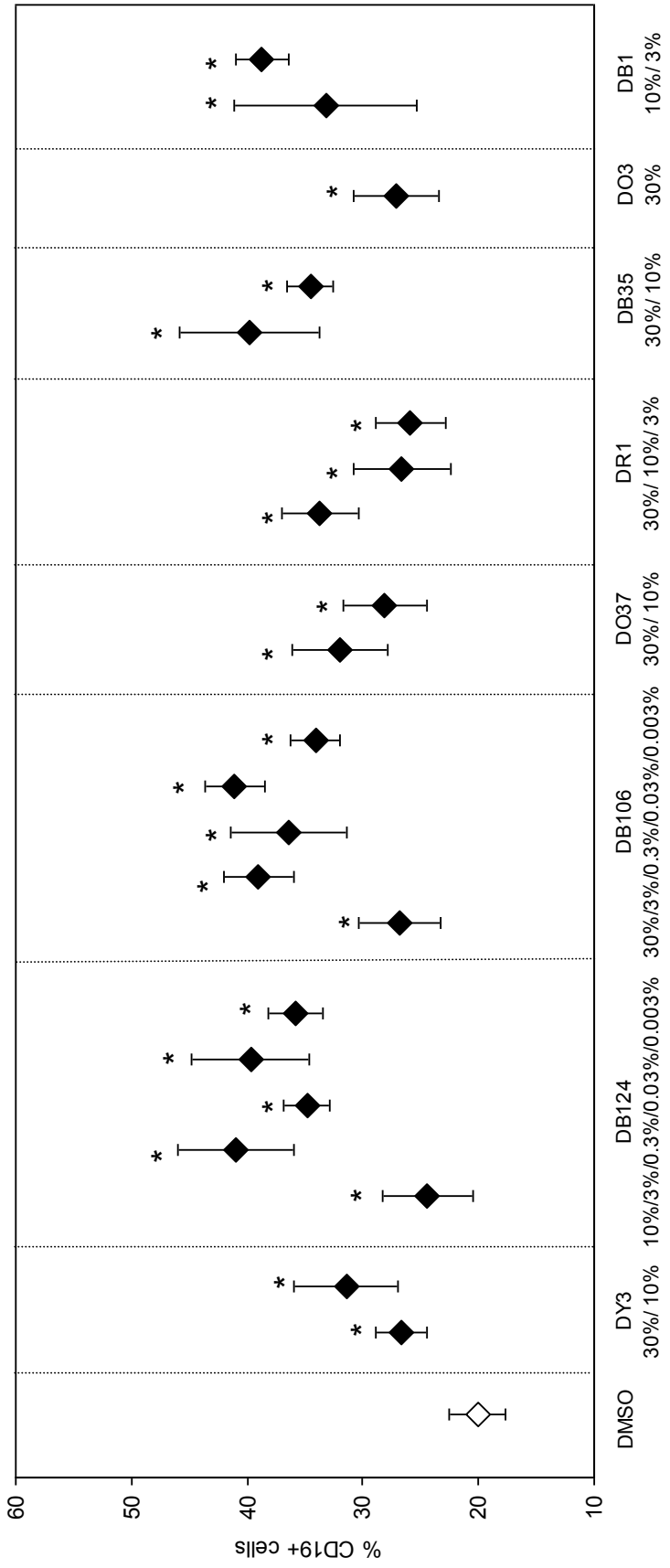


Fig. 3.1.5.3 CD19+ cell population (mean ± SD) from lymph nodes in the biphasic LLNA protocol. *Significant increase at $p<0.05$ (t-test). Minimum value on the y-axis is not 0, to give a better visual cpmparison of the results between various groups

Disperse blue 1 application at 10 and 3% concentrations caused a significant enhancement in CD19+ lymphocytes by 13 and 19%, respectively, in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.5.3).

3.1.5.4 CD45+ cell population

Disperse yellow 3 treatment at 30 and 10% concentrations showed no significant changes in the CD45+ cells between the treated and the control animals (Fig. 3.1.5.4).

In the experiment with Disperse blue 124 at various concentrations, a significant increase in CD45+ cell population by 16, 27 and 23%, respectively, was observed in the mice treated with 10, 3 and 0.03% concentrations as compared to the vehicle treated control mice (Fig. 3.1.5.4). At 0.3 and 0.003% concentrations, a non-significant increase of 10 and 3%, respectively, was seen in the CD45+ cells in the treated animals.

Application of Disperse blue 106 at 30, 3, 0.3 and 0.03% concentrations resulted in a significant enhancement in the CD45+ epitope marker by 17, 29 and 22% at 30, 3 and 0.03% concentrations, respectively; while at 0.3% concentration a non-significant increase of 8% was observed in the treated animals in comparison to the DMSO treated control animals. At 0.003% concentration, no relevant difference was observed (Fig. 3.1.5.4).

Application of Disperse orange 37 at 30 and 10% concentrations showed a non-significant increase in the CD45+ cells by 8 and 7%, respectively, in the treated mice as compared to the vehicle control mice (Fig. 3.1.5.4).

In the study involving Disperse red 1 treatment at 30, 10 and 3% concentrations, no relevant modulation was found in the CD45+ cell surface marker between the treated and control animals (Fig. 3.1.5.4).

Disperse blue 35 application at 30 and 10% concentrations caused a significant increase in the CD45+ lymphocytes by 12 and 21%, respectively, in the treated animals in comparison to the DMSO treated control animals (Fig. 3.1.5.4).

No significant modulation was seen in the CD45+ cell population in the mice following Disperse orange 3 treatment at 30% concentration, in comparison to the vehicle treated mice (Fig. 3.1.5.4).

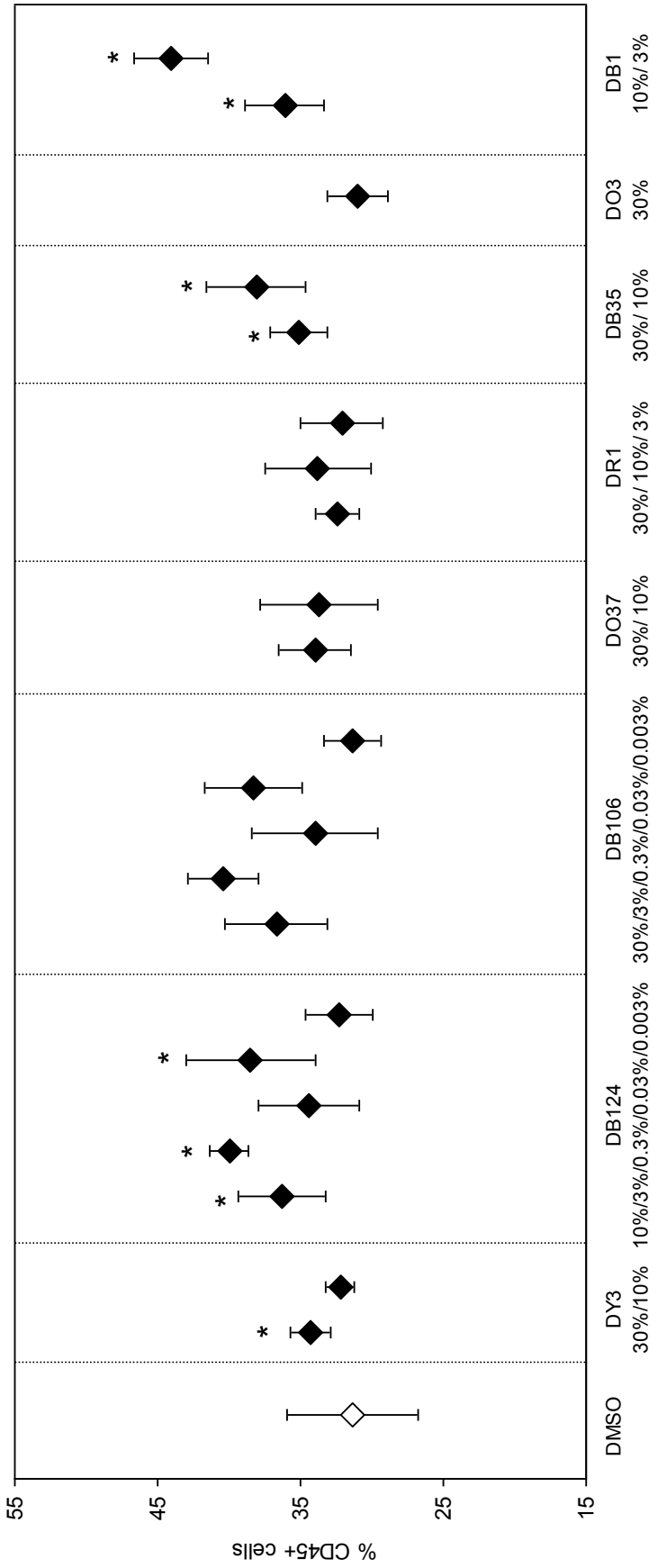


Fig. 3.1.5.4 CD45+ cell population (mean ± SD) from lymph nodes in the biphasic LLNA protocol, *Significant increase at $p<0.05$ (t-test). Minimum value on the y-axis is not 0, to give a better visual comparison of the results between various groups.

Treatment with Disperse blue 1 at 10 and 3% concentrations led to a significant increase in the CD45+ cell surface marker by 15 and 40%, respectively, in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.5.4).

3.1.5.5 CD45+1A cell population

Mice treated with Disperse yellow 3 at 30 and 10% concentrations showed an increase in the CD45+/1A+ cells by 48 and 35%, respectively, in comparison to the DMSO treated control animals (Fig. 3.1.5.5).

In the experiment involving application of Disperse blue 124 at 10, 3, 0.3, 0.03 and 0.003% concentrations, a significant increase in the CD45+/1A+ cell surface marker by 45, 98, 43, 50 and 43%, respectively, was seen in the treated animals as compared to the vehicle control animals (Fig. 3.1.5.5).

Application of Disperse blue 106 at 30, 3, 0.3, 0.03 and 0.003% concentrations showed a significant increment in the CD45+/1A+ cells by 52, 90, 45, 63 and 46%, respectively, in the treated mice in comparison to the vehicle control mice (Fig. 3.1.5.5).

Treatment with Disperse orange 37 at 30 and 10% concentrations resulted in a significant modulation in the CD45+/1A+ lymphocytes by +75 and +43%, respectively, in the treated mice as compared to the DMSO treated control mice (Fig. 3.1.5.5).

Disperse red 1 application at 30 and 10% concentrations caused a significant increase in the CD45+/1A+ epitope marker by 80 and 29%, respectively, while at 3% concentration a non-significant modulation of +12% was observed in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.5.5).

Following Disperse blue 35 application at 30 and 10% concentrations, a significant increase in the CD45+/1A+ cell population by 111 and 50% was observed in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.5.5).

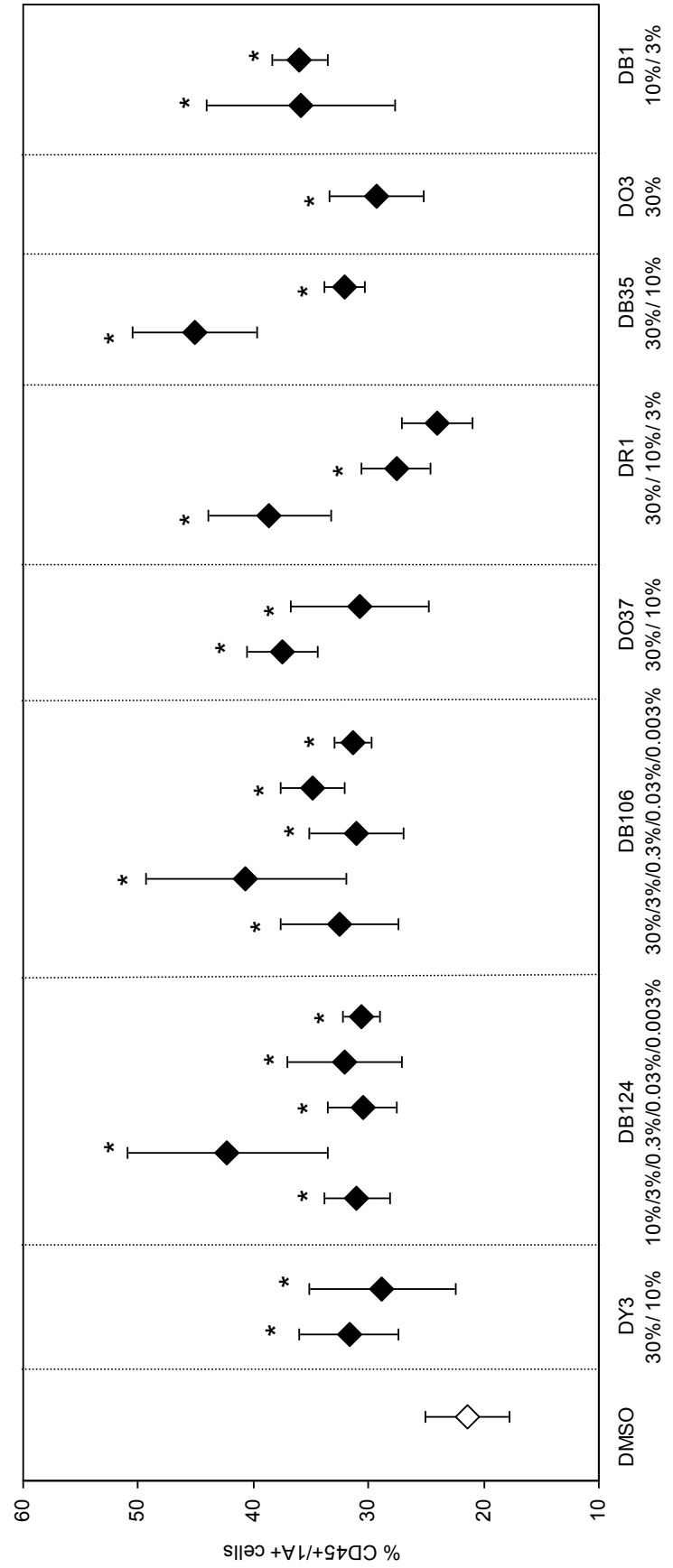


Fig. 3.1.5.5 CD45+/1A+ cell population (mean ± SD) from lymph nodes in the biphasic LLNA protocol. *Significant increase at p<0.05 (t-test). Minimum value on the y-axis is not 0, to give a better visual comparison of the results between various groups.

A significant increase of 37% was seen in the CD45+/1A+ cells in the mice treated with Disperse orange 3 at 30% concentration as compared to the vehicle control mice (Fig. 3.1.5.5).

The CD45+/1A+ population showed a significant increase by 68% in the mice treated with 10 and 3% concentrations of Disperse blue 1, in the treated mice in comparison to the vehicle treated control mice (Fig. 3.1.5.5).

3.1.5.6 CD4+/CD69+ cell population

Treatment with Disperse yellow 3 at 30% concentration showed a significant increase in the CD4+/CD69+ cells by 24%, while at 10% concentration no relevant difference was observed in the treated mice as compared to the vehicle control mice (Fig. 3.1.5.6).

Disperse blue 124 treatment at 10, 3 and 0.3% concentrations showed a significant increase in the CD4+/CD69+ cells by 56, 28 and 32%, respectively, while a non-significant increase of 4% was observed at 0.03% concentration in the treated mice as compared to the DMSO treated control mice. No relevant difference was observed when the mice were treated with 0.003% concentration (Fig. 3.1.5.6).

Application of Disperse blue 106 at 30, 3 and 0.3% concentrations led to a significant enhancement in the CD4+/CD69+ cell population by 40, 35 and 16%, respectively, whereas a non-significant enhancement of 5 and 4% was observed at 0.03 and 0.003% concentrations, respectively, in the treated animals as compared to the vehicle treated control animals (Fig. 3.1.5.6).

Disperse orange 37 treatment at 30 and 10% concentrations did not cause any relevant changes in the CD4+/CD69+ cells between the treated and control animals (Fig. 3.1.5.6).

Treatment with 30, 10 and 3% concentrations of Disperse Red 1 did not result in any changes in the CD4+/CD69+ cells in the treated mice in comparison to the vehicle control mice (Fig. 3.1.5.6).

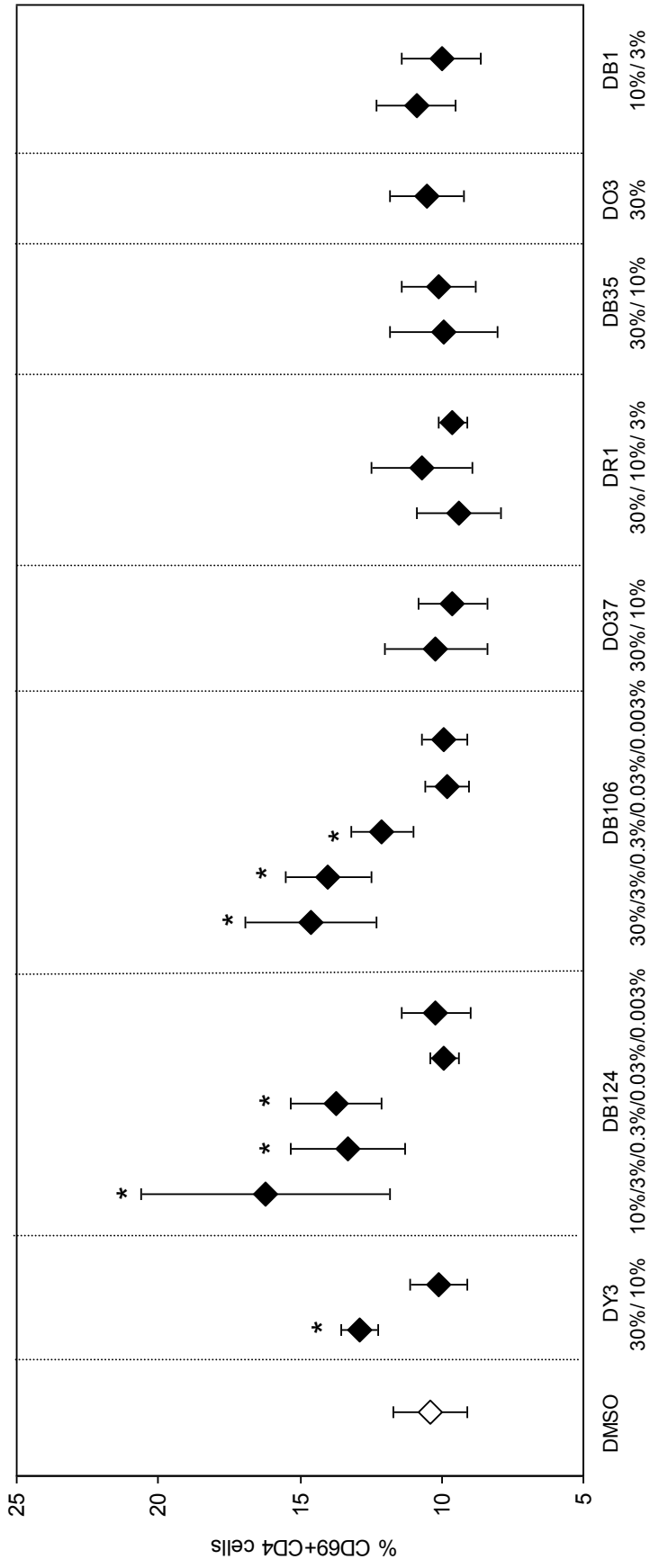


Fig. 3.1.5.6 CD69+/CD4+ cell population (mean ± SD) from lymph nodes in the biphasic LLNA protocol. *Significant increase at $p<0.05$ (t-test). Minimum value on the y-axis is not 0, to give a better visual comparison of the results between various groups

No significant difference was observed in the CD4+/CD69+ cell population in the mice treated with Disperse blue 35 at 30 and 10% concentrations, as compared to DMSO treated control mice (Fig. 3.1.5.6).

Disperse orange 3 treatment at 30% concentration caused no modulation in the CD4+/CD69+ lymphocytes in the treated animals as compared with control animals (Fig. 3.1.5.6).

Following application of 10 and 3% solution of Disperse blue 1, no relevant change was seen in the CD4+/CD69+ cell population between the treated and the control mice (Fig. 3.1.5.6).

Table 3.1.5.1 gives the combined results for flow cytometry of all the textile dyes tested at various concentrations.

3.2 LCSA

All the textile dyes were tested at various concentrations for their cytotoxicity. The CD86 expression was measured at the concentrations at which a significant number of DCrc remain viable. The half-maximal increase in CD86 expression (EC50) values were used to compare the sensitising potency values of the substances tested.

Disperse blue 124 was tested at 0.1 µM, 0.5 µM, 1 µM, 3 µM, 6.5 µM and 12 µM concentrations. The relative CD86 expression at these concentrations is shown in Fig. 3.2.1.

The various concentrations tested for Disperse yellow 3 were 1 µM, 5 µM, 10 µM, 20 µM, 25 µM and 50 µM. The CD86 expression at the tested concentrations is displayed in Fig. 3.2.2.

The textile dye Disperse blue 1 was tested at 1 µM, 5 µM, 10 µM, 25 µM, 50 µM and 100 µM. Fig. 3.2.3 shows the CD86 expression at the tested concentrations.

Disperse red 1 was tested at 1 µM, 5 µM, 10 µM, 20 µM and 25 µM concentrations. The CD86 expression at the various concentrations tested is shown in Fig. 3.2.4.

The compound Disperse orange 3 was tested at 1 µM, 10 µM, 25 µM, 50 µM and 100 µM. Fig. 3.2.5 displays the CD86 expression at the tested concentrations.

Table 3.1.5.1 Phenotypic analysis (% positive cell population, Mean±SD) of the different epitope markers on lymphocytes obtained from lymph nodes of mice treated with various disperse dyes according to the sensitisation-challenge protocol.

		CD4+	CD8+	CD19+	CD45+	CD45+/1A+	CD69+/CD4+
1	Vehicle control	47.7±4.3	20.8±2.1	20.1±2.5	31.4±4.0	21.4±3.4	10.3±1.3
2	DY3 30%	43.8±5.6	21.2±3.1	26.7±2.5*	34.3±1.7*	31.7±3.2*	12.9±0.79*
	DY3 10%	43.5±2.0*	21.0±2.6	31.4±4.5*	32.2±1.0	28.8±6.4*	10.1±1.0
3	DB124 10%	40.7±4.1*	18.3±1.7*	24.4±3.9*	36.3±3.1*	31.0±2.9*	16.2±4.4*
	DB124 3%	37.4±5.5*	15.6±3.4*	41.0±5.0*	40.0±1.3*	42.3±8.7*	13.3±2.0*
	DB124 0.3%	39.6±2.4*	18.7±2.0*	34.8±2.0*	34.4±3.5	30.5±3.0*	13.7±1.6*
	DB124 0.03%	38.8±3.8*	15.4±2.0*	39.7±5.1*	38.5±4.5*	32.1±5.0*	9.9±0.52
	DB124 0.003%	40.6±1.5*	15.5±1.1*	35.8±2.4*	32.3±2.3	30.6±1.6*	10.2±1.2
4	DB106 30%	39.6±3.3*	17.4±2.4*	26.8±3.5*	36.7±3.6*	32.5±5.1*	14.6±2.3*
	DB106 3%	38.4±3.5*	16.6±3.6*	39.0±3.0*	40.4±2.5*	40.7±8.7*	14.0±1.5*
	DB106 0.3%	38.5±3.7*	19.0±2.2*	36.4±5.0*	34.0±4.4	31.1±4.1*	12.1±1.1*
	DB106 0.03%	36.0±1.6*	16.3±1.6*	41.1±2.6*	38.3±3.4*	34.9±2.8*	9.8±0.78
	DB106 0.003%	40.4±1.2*	15.1±1.3*	34.1±2.1*	31.3±2.0	31.3±1.6*	9.9±0.82
5	DO37 30%	32.0±4.1*	15.4±2.3*	32.0±4.1*	34.0±2.5	37.5±3.1*	10.2±1.8
	DO37 10%	41.7±4.1*	21.0±2.2	28.1±3.6*	33.7±4.1	30.7±6.0*	9.6±1.2
6	DR1 30%	36.1±5.2*	16.0±1.7*	33.7±3.3*	32.4±1.5	38.6±5.3*	9.4±1.5
	DR1 10%	43.4±2.5*	21.6±1.7	26.6±4.2*	33.8±3.7	27.6±3.0*	10.7±1.8
	DR1 3%	46.1±3.3	20.2±1.5	25.9±3.0*	32.1±2.9	24.0±3.1	9.6±0.50
7	DB35 30%	36.3±4.7*	16.7±2.0*	39.8±6.1*	35.1±2.0*	45.1±5.4*	9.9±1.9
	DB35 10%	40.0±2.1*	19.3±1.8*	34.5±2.0*	38.1±3.5*	32.1±1.8*	10.1±1.3
8	DO3 30%	44.0±3.3*	19.1±1.4*	27.1±3.7*	31.0±2.1	29.3±4.1*	10.5±1.3
9	DB1 10%	40.2±3.0*	17.1±1.7*	33.2±7.9*	36.1±2.8*	35.9±8.2*	10.9±1.4
	DB1 3%	40.0±2.1*	17.4±2.0*	38.7±2.3*	44.1±2.6*	36.0±2.4*	10.0±1.4

Sample size was n=10 in all the treated groups, n=20 for the vehicle control group. DMSO was used as vehicle. DY3 Disperse yellow 3, DB124 Disperse blue 124, DB106 Disperse blue 106, DO37 Disperse orange 37, DR1 Disperse red 1, DB35 Disperse blue 35, DO3 Disperse orange 3, DB1 Disperse blue 1. ***** Indicate significant change at $p<0.05$ (t-test) between vehicle control and treated animals

Disperse blue 35 was tested at 10 µM, 25 µM, 50 µM and 100 µM. The CD86 expression at the various concentrations tested is displayed in Fig. 3.2.6.

The textile dye, Disperse blue 106, was tested at 5 µM, 10 µM and 12.5 µM. Fig. 3.2.7 shows the CD86 expression at the different tested concentrations.

Disperse orange 37 0.5 µM, 5 µM, 25 µM, 50 µM and 100 µM concentrations. The CD86 expression at various concentrations has been displayed in Fig. 3.2.8.

The half-maximum increase in CD86 expression, the EC50 value, was calculated for all the substances tested (Table 3.2.1).

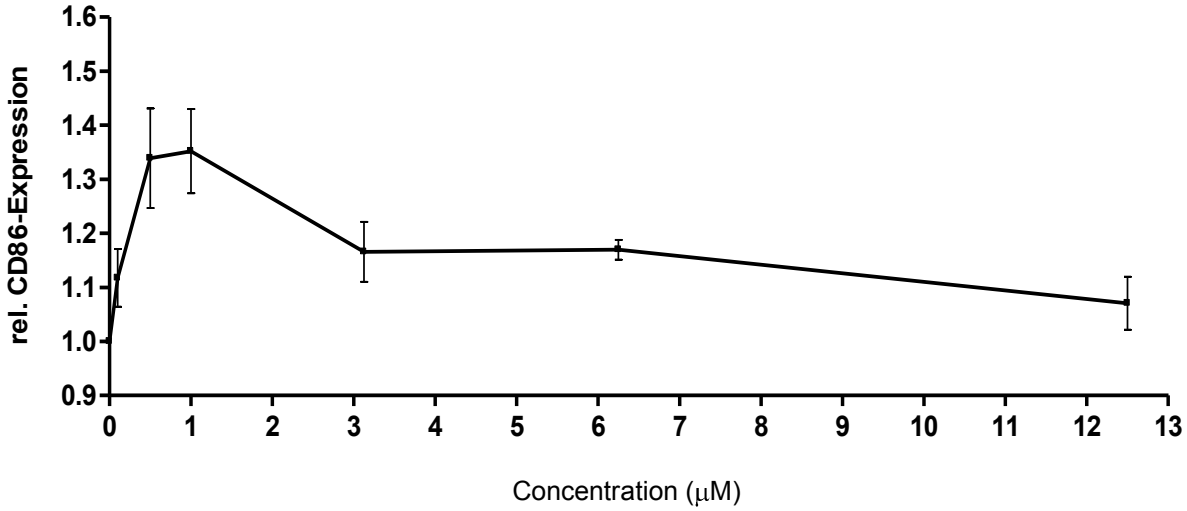


Fig. 3.2.1 CD86 expression of DCrc following exposure to DB124 at various concentrations

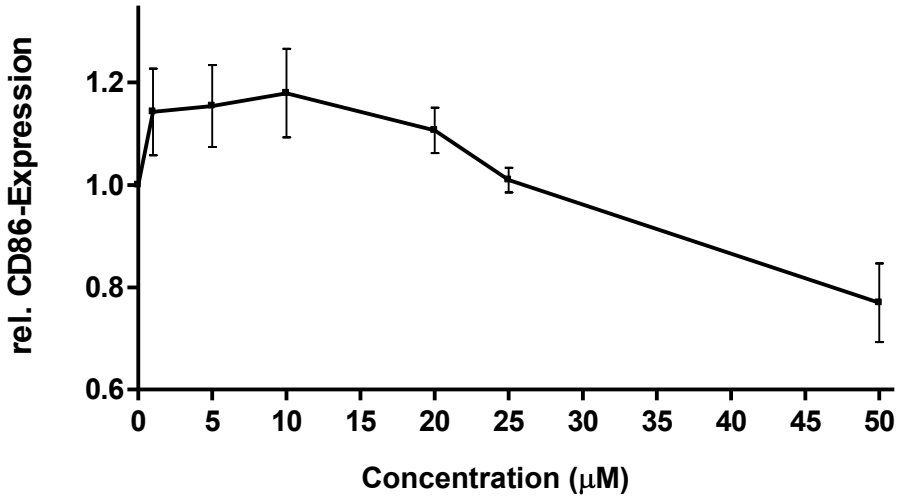


Fig. 3.2.2 CD86 expression of DCrc following exposure to DY3 at various concentrations

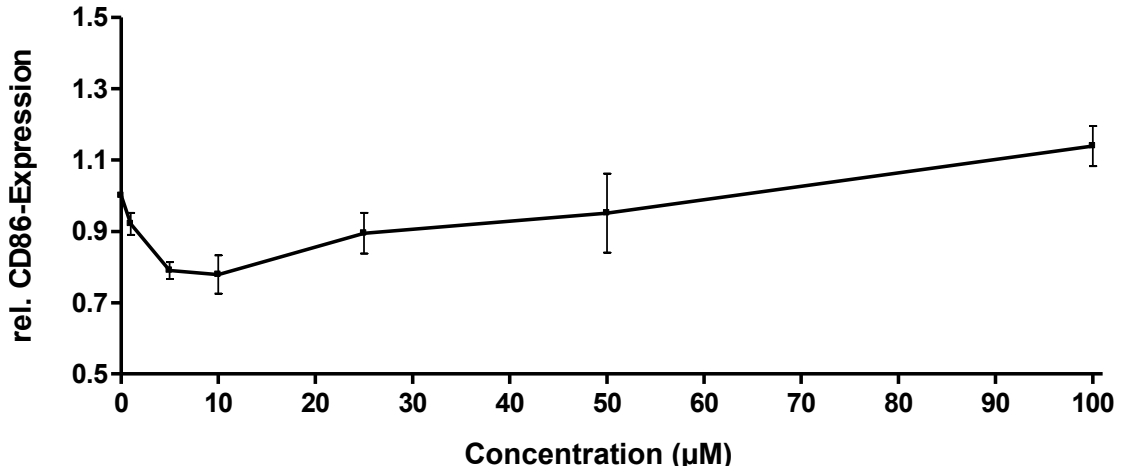


Fig. 3.2.3 CD86 expression of DCrc following exposure to DB1 at various concentrations

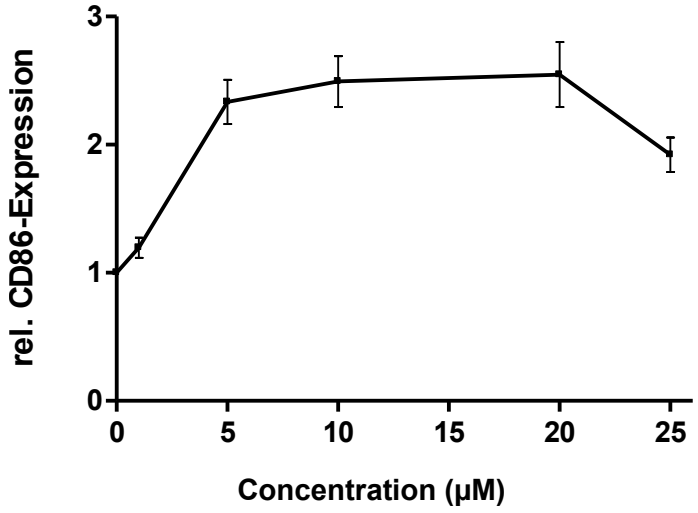


Fig. 3.2.4 CD86 expression of DCrc following exposure to DR1 at various concentrations

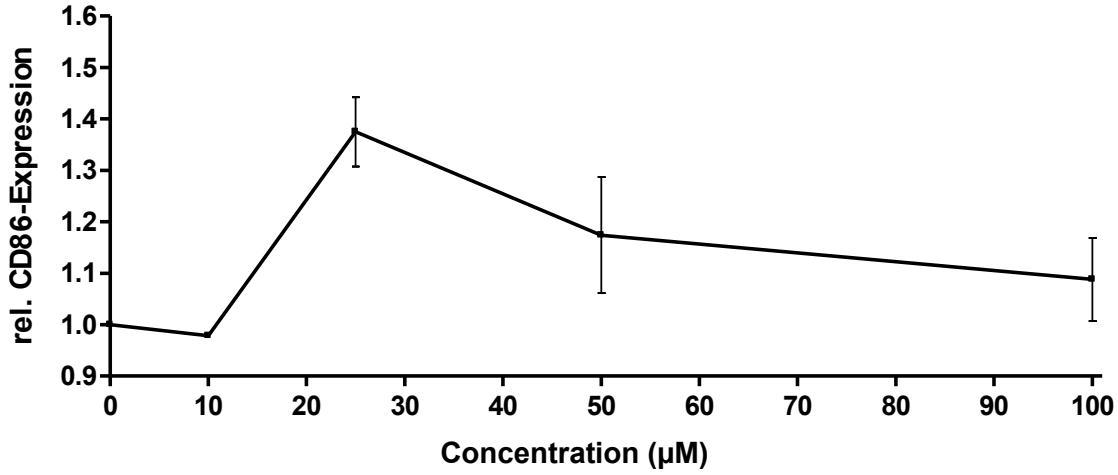


Fig. 3.2.5 CD86 expression of DCrc following exposure to DO3 at various concentrations

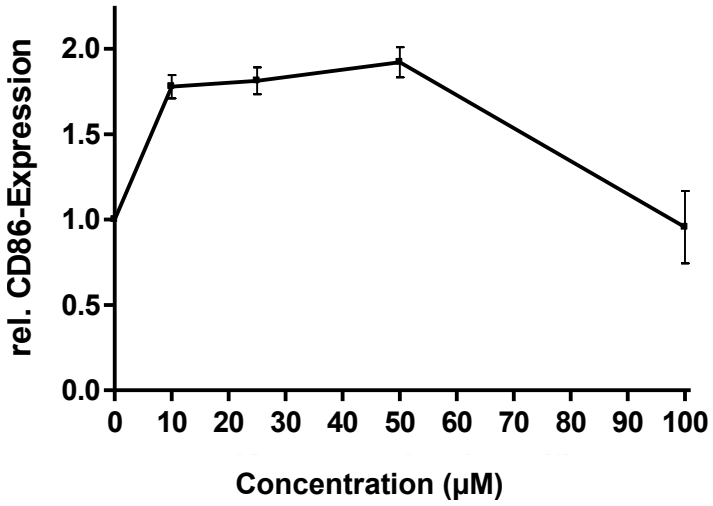


Fig. 3.2.6 CD86 expression of DCrc following exposure to DB35 at various concentrations

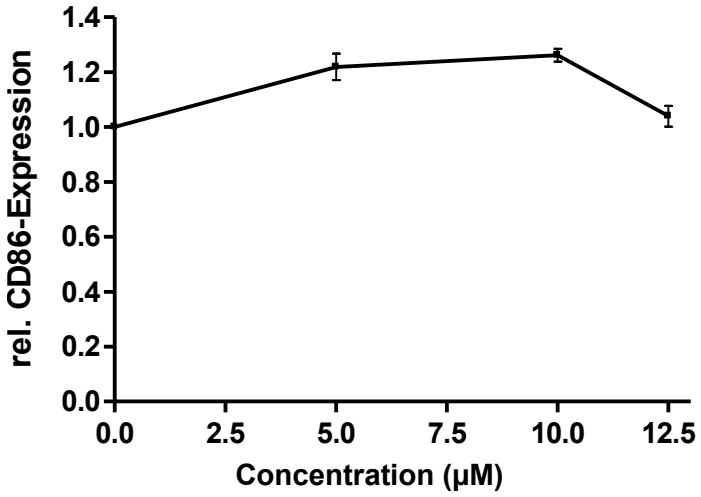


Fig. 3.2.7 CD86 expression of DCrc following exposure to DB106 at various concentrations

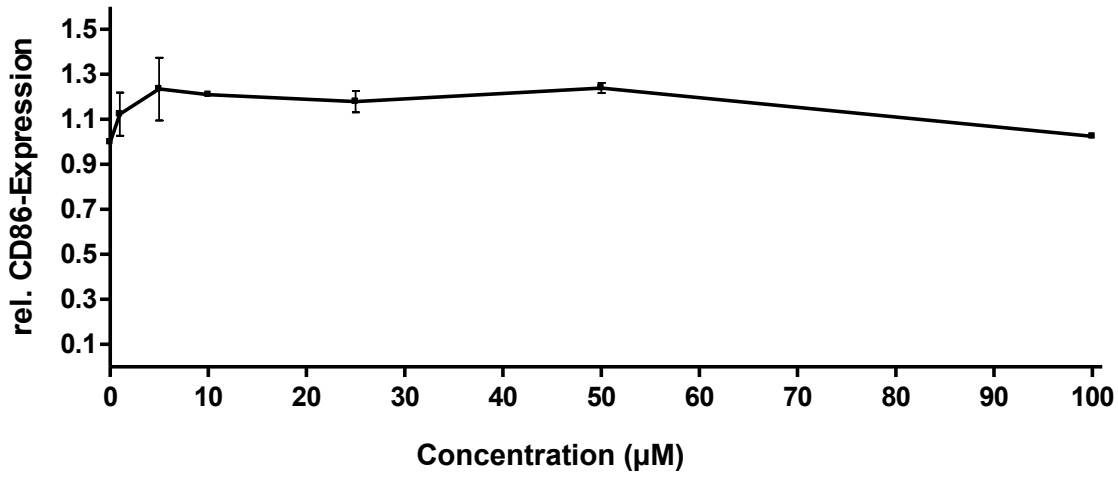


Fig. 3.2.8 CD86 expression of DCrc following exposure to DO37 at various concentrations

Substance	Sensitisation EC50 / µM
Disperse blue 1	-
Disperse blue 35	6
Disperse blue 106	2.5
Disperse blue 124	0.25
Disperse orange 3	18
Disperse orange 37	1
Disperse red 1	3
Disperse yellow 3	0.5

Table 3.2.1 EC50 values, the half-maximal increase in CD86 expression, calculated for the various textile dyes

4 Discussion

4.1 Anatomical endpoints of the LLNA

The LLNA is based on the observations of lymph node cell proliferation and local lymph node enlargement during the induction phase of allergy (Oort and Turk, 1965; Parrott and de Sousa, 1966; Asherson and Barnes, 1973). The OECD validated LLNA protocol involves the use of radioactive labelling with ^{3}H-thymidine (OECD Test Guideline no.429, 2002). The *in vivo* incorporation of thymidine into lymphocytes is a measure of the proliferation of lymphocytes, an event in sensitisation (Kimber et al., 1989). This endpoint is used to calculate the stimulation index (SI), followed by EC3 calculation (the concentration of a chemical which causes 3 times proliferation in the treated mice as compared to the control) which is considered to be a safe criterion for the identification and classification of chemicals with allergenic effects. The accuracy of this criterion was established following comparison of results from different international laboratories using different substances (Basketter et al., 1999b).

Besides the practical problems with the handling of radioactive materials and contaminated animals, the validated LLNA protocol also showed inconsistencies in the identification of weak allergens and the distinction between irritant and allergenic effects of a substance. The allergen nickel failed to provoke positive response in the LLNA, and also in other animal tests i.e. the GMPT. Nickel has a weak allergenic potential, along with wide dissemination and usually long duration of exposure (Kimber et al., 2002). On the other hand, several working groups observed that some phototoxic irritant substances also cause an increase in cell proliferation in lymph nodes and thus showed false positive results (Basketter et al., 1998; Kimber et al., 2002; Montelius et al., 1994; Scholes et al., 1992). Irritants such as sodium dodecyl sulphate (SDS), oxalic acid, triton X-100 and methylsalicylate gave positive results in LLNA, which were not distinguishable from those of weak and moderate allergens (Montelius et al., 1994). Limitations of various tests for predicting sensitizing properties of compounds i.e. the guinea-pig maximization test, Buehler occluded patch test and the LLNA have been reviewed by Basketter and Kimber (2007).

Therefore, many groups have worked on alternative endpoints, in an effort to increase the sensitivity of the LLNA and to increase its practical implementation by the omission of radioactivity from the protocol (Vohr et al., 1994; Homey et al., 1998; Ulrich et al., 1998). Despite various efforts, only one of the alternative end points, lymph node cell count, has yet

been evaluated thoroughly in intra- (Vohr et al., 2000) and inter-laboratory trials (Ehling et al., 2005a,b). These studies included a comparison between [^{3}H]-thymidine incorporation and cell counts along with measurement of the ear thickness and ear weight. The results confirmed similar sensitivity between both methods. Besides avoiding radioactivity, determining cell counts instead of thymidine incorporation has the advantage that cell suspension can be used for further analysis with various methods (flow cytometry, chemiluminescence and immunofluorescence) in order to study mechanistic events (Ikarashi et al., 1993; Vohr et al., 1994; Gerberick et al., 2002; Ulrich et al., 2001; Yamashita et al., 2005).

4.1.1 Lymph node cell number and weight

LLNA focuses on the changes in the lymph drainage of the skin by the trigger of contact allergy. These include changes such as increase in lymph node weight and the increase in the rate of lymphocyte cell division and cell number in the lymph nodes. This led to the development of endpoints such as lymph node cell number and weight as very sensitive and stable markers for the identification of allergenic substances (Homey et al., 1998; Ulrich et al., 1998; Vohr et al., 2000; Ehling et al., 2005a,b). These endpoints showed a similar sensitivity as the measurement of the incorporation of radioactive thymidine into the lymphocytes (Basketter and Scholes, 1992; Dearman et al., 1999; Loveless et al., 1996). In addition, some weak allergens such as eugenol and mercaptobenzothiazole were also correctly identified (Ulrich et al., 2001).

In our study, the lymph node cell number and lymph node weight proved to be sensitive and reliable end-points. Almost all the dyes except Disperse orange 3 led to a significant increase in cell count and lymph node weight at various concentrations tested. Disperse blue 124 and Disperse blue 106 showed the most pronounced increase in both the parameters. Disperse blue 124 at 10% concentration resulted in increase in cell count by 147%, while the lymph node weight increased by 139% as compared to the control group. Almost in all the experiments, we observed a significant increase in the lymph node cell count associated with an increase in the lymph node weight, these changes mostly being dose-dependent. Some other working groups have reported that the increase in cell number was significant with moderate allergens, while the weight of lymph nodes showed only a slight tendency to increase (Homey et al., 1998; Vohr et al., 2000).

Based on our results, the disperse dyes could be arranged in four groups on the basis of their sensitising potency in the following decreasing order (in parenthesis: lowest concentration causing a significant increase in lymph node cell number): group 1, strong: Disperse blue 124 and Disperse blue 106 (0.003%), group 2, moderate: Disperse red 1 and Disperse blue 1 (3%), group 3, weak: Disperse orange 37 and Disperse blue 35 (10%) and group 4, very weak: Disperse yellow 3 and Disperse orange 3 (increase at 30% or no increase at 30%).

In studies from other research groups, the cell count method has been found to be at least as sensitive as the radioactive method using ^{3}H-thymidine incorporation to evaluate the cell proliferation (Suda et al., 2002; Homey et al., 1998; Vohr et al., 2000; Ulrich et al., 2001). An interesting aspect in the measurement of the lymph node weight and cell number in lymph nodes is that these changes are predictive of the whole treatment period. They reflect the outcome of all the cellular changes such as proliferation, migration and apoptosis of lymphocytes in lymph nodes. On the other hand, measurement of radioactive thymidine presents only a limited time window of the changes. It is like a snapshot of cell proliferation between the last treatment and the measurement of the incorporated thymidine (Ulrich et al., 2001).

The problem in distinguishing between allergens and irritants exist also in the use of alternative endpoints, cell number and lymph node weight. Several working groups showed that even irritants and phototoxic substances can generate significant lymph node activation in the LLNA (Gerberick et al., 1992; Homey et al., 1995; Ikarashi et al., 1993; Scholes et al., 1991; Vohr et al., 1994; Ulrich et al., 2001). Homey and coworkers observed a dose-dependent increase in cell number and weight of lymph nodes by the irritant croton oil (Homey et al., 1998). This increase was similarly pronounced as after treatment with the strong allergen oxazolone. Also, Ulrich and coworkers observed similar effects for croton oil on the lymph nodes as for the contact allergens DNCB and oxazolone (Ulrich et al., 2001).

Due to similarity in clinical and histological features of allergic and irritant contact dermatitis, the differentiation between both types of dermatitis in the preclinical and clinical evaluation of chemicals remains difficult (Lachapelle, 1997). However, the primary immunological mechanisms are considered to be fundamentally different. The induction of a specific cytokine with repeated exposure to allergen to previously sensitized lymphocytes has been used to differentiate between allergens and irritants. Contact allergens induce marked induction of proinflammatory cytokines such as IL-1α, IL-1β, macrophage inflammatory protein-2 (MIP-2), IFN-induced protein-10 (IP-10), TNF-α and GM-CSF along with T helper

type 1 (Th1; IL-2, IFN-γ) and type 2 (Th2; IL-4, IL-10) cytokines. However, irritants showed marginal upregulation of IL-1α, IL-6, TNF-α and IFN-γ (Ulrich et al., 1998; Enk and Katz, 1992; Kondo et al., 1994; Homey et al., 1998; Fehr et al., 1994; Hope et al., 1994a,b). Dearman and coworkers proposed that there are also differences in the cytokine pattern between contact allergens and respiratory allergens (Dearman et al., 1994). Since the cell proliferation in lymph nodes is associated with the increase of Langerhans cells, it is assumed that the cytokine induction by irritation also causes migration of Langerhans cells in the lymph nodes (Kimber et al., 1994a,b; Cumberbatch et al., 1993; Cumberbatch and Kimber, 1995; Kinnaird et al., 1989).

Allergic skin reaction involves antigen presentation and T-cell activation followed by the formation of antigen-specific memory T-cells. Whereas, the immune cascade is triggered after contact of skin with an irritant and inflammatory mediators and cytokines are released, thus involving a non-specific T-cell activation effect (Enk and Katz, 1995; Hunziker et al., 1992; Kondo et al., 1994).

4.1.2 Changes in the ears

It was shown that the increase in ear thickness caused by the inflammation of the skin due to substances with irritant effect is a useful marker for the LLNA and varies in allergic and irritant reactions (Homey et al., 1998; Vohr et al., 2000). These researchers showed that the irritant croton oil caused significant ear swelling, while the allergen oxazolone showed only a slight effect on ear-thickness, the observations being concentration-dependent. Treatment with 0.3% oxazolone resulted in non-significant ear swelling along with a distinct increase in lymph node cell count. However, 1% oxazolone induced marginal but significant skin inflammation, which was associated with a predominant increase in lymph node cell counts. Treatment with 0.3% croton oil showed significant skin inflammation and a marginal but significant lymph node cell proliferation. Marked ear swelling was detected after 1% croton oil administration, accompanied with significantly increased lymph node cellularity. They concluded that in comparison with chemical-induced lymph node cell proliferation, contact allergens induce only marginal skin inflammation; however, irritant chemicals induce marked skin inflammation.

Ulrich and coworkers observed that oxazolone and croton oil have similar effects on cell number and the weight of the lymph nodes (Ulrich at al., 2001). To distinguish the effects of these substances, they measured the weight of the ears using a punch from the ear and

observed that the irritation caused by inflammation of the skin had a stronger influence on the weight of the ears, as compared to the inflammation triggered by allergic reactions. Also, the croton oil-treated ears showed a significant weight gain after 24 h and 48 h, which increased further after 72 h and reached a plateau. The allergen oxazolone increased the weight of the ears after 48 h and 72 h, but could not achieve the level of the croton oil produced effect. In the mouse-ear swelling test (MEST), known allergens also have been reported to cause inflammation of the skin (Gad et al., 1986).

In the present study, changes in both ear-thickness and ear punch-weight were observed. Among the various textile dyes studied, the most significant increase in ear-thickness and ear punch-weight were observed with Disperse blue 124 and Disperse blue 106. At 0.3% concentration, Disperse blue 124 resulted in an increase of the ear-thickness and ear-punch weight by 30 and 28%, respectively, which was the highest in comparison to the other concentrations tested. Disperse blue 106 at 30% concentration resulted in an increase of ear-thickness by 26%, while the ear-punch weight was increased by 22%, the increase being the most significant in contrast to the other concentrations tested. All the other tested textile dyes affected the ear parameters only moderately. The effect on ear-thickness and ear punch-weight was not found to be dose-dependent in all the textile dyes tested. The low impact on the ear parameters together with the marked changes in cell number and weight of the lymph nodes reflects mainly the allergenic effects of textile dyes tested in the present study.

Another approach to limit the false positive results by irritant effects of the substances is to examine the lymphocyte fractions in the local lymph nodes. Several working groups measured lymphocyte surface epitopes (Ulrich et al., 1998; Vohr et al., 1994) by using flow cytometry so that allergens could be successfully distinguished (Homey et al., 1998).

4.2 Cellular changes in lymph nodes

In the present study, percentages of CD4+, CD8+, CD19+, CD45+, CD45+/1A+, CD4+/CD69+ cells were analysed using flow-cytometry. CD4 is an antigen coreceptor on the T-cell surface which interacts with MHC class II molecules on antigen-presenting cells. It participates in T-cell activation through its association with the T-cell receptor complex and protein tyrosine lck (Janeway, 1992). CD4+ cells are designated as T-helper cells. CD8 is an antigen coreceptor on the T-cell surface which interacts with MHC class I molecules on antigen-presenting cells or epithelial cells. It participates in T-cell activation through its

association with the T-cell receptor complex and protein tyrosine kinase lck (Zamoyska, 1994). CD8+ cells are known as T-cytotoxic cells. CD19, a 95kDa transmembrance glycoprotein, is a member of the immunoglobulin superfamily and is expressed throughout B-lymphocyte development from the pro-B cell through the mature B-cell stages (Krop et al., 1996). CD45 is expressed on B lymphocytes at all stages from pro-B through mature and activated B cells, but it is decreased on plasma cells and a subset of memory B cells (Hathcock et al., 1992). CD69 expression is rapidly induced upon activation of lymphocytes (T, B, NK, and NK-T cells), neutrophils, and macrophages (Ziegler et al., 1994). CD69, also known as “very early antigen”, is an indicator of lymphocyte activation. I-A/I-E is a MHC class II molecule and is expressed on antigen presenting cells (including B cells) and a subset of T cells and is involved in antigen presentation to T cells (Bhattacharya et al., 1981). CD4+/CD69+ and CD45+/1A+ represent activated T-helper and B cells, respectively.

One limitation of the LLNA is the problem of lymphocyte proliferation in the investigation of substances with an irritant effect (Montelius et al., 1994; Gerberick et al., 1992). Homey and coworkers showed that allergens and irritants can be differentiated by the changes in surface epitope markers CD45, CD69 and 1A. The results of Homey and coworkers were confirmed by Gerberick and coworkers (Homey et al., 1998; Gerberick et al., 1999), also noticing that the irritant benzalkonium chloride is misinterpreted as allergen in the LLNA using the cell proliferation values (Gerberick et al., 1992). Sikorski and coworkers showed that allergens and irritants could be correctly identified based on the changes in the relationship between B and T lymphocytes (Sikorski et al., 1996). Using the SI 3 as a criterion for an allergic effect, they observed that 10 out of 16 irritants gave false positive results. It was only through further analysis of surface epitope markers for lymphocytes that some irritants could be properly identified (Sikorski et al., 1996).

In the present work, the allergenic effect of the substances investigated was confirmed by the change in lymphocyte subpopulations. The selection of used antibodies against different surface molecules of T-lymphocytes and B-series were based largely on the work by Homey, Sikorski, Gerberick and coworkers (Homey et al., 1998; Sikorski et al., 1996; Gerberick et al., 2002). These effects observed in our experiments have also been described earlier (Sikorski et al., 1996; Hariya et al., 1999; Stahlmann et al., 2006; Ahuja et al., 2009 a,b).

4.2.1 T lymphocytes

The changes of T cell fraction in allergic reactions of the delayed type are the focus of many

investigations. In the study by Homey and coworkers, topical treatment with 1% oxazolone markedly induced both CD25 and CD69 on CD4+ and CD8+ lymph node cells, while no such induction could be seen following croton oil treatment (Homey et al. 1998). Also, treatment with oxazolone resulted in significant expansion of I-A+/B220+ and CD69+/I-A+ lymph node cells. While, treatment with croton oil showed only slight increase in I-A+/B220+ and CD69+/I-A+ lymph node cell population. They concluded that flow cytometric analysis of lymph node cell populations provides additional evidence for differentiation between allergic and irritant skin reactions. However, they also added that the findings of their study were based on experiments with two selected allergens and irritants, therefore, further studies with broad range of chemicals should be carried out.

The results of the present study showed that the relative proportion of CD4+ and CD8+ cells in the treated animals decreased in comparison to the control animals. The proportion of CD19+, CD45+ and CD45+/1A+ cells increase in almost all the treated animals as compared to the control animals. These changes are in agreement with the observations of Sikorski and coworkers (Sikorski et al., 1996). They noticed that both the allergens and the irritants produced an increase in cell number per node in comparison to the vehicle control. The increase in cell number was reflected as an increase in the total number of CD3+, B220+, IgG+ and IgM+, CD4+ and CD8+ cells. However, estimation of the percentage of the various subpopulations showed that allergen exposure altered the percentages of certain subpopulations to a larger extent than irritants. Most noticeable was the increase in the number of B lymphocytes consistently seen in the allergen treated mice over vehicle control. They found that the percentages of CD3+, CD4+ and CD8+ cells generally dropped in the allergen treated mice because of the associated increase in B lymphocytes. The increase in CD69+/CD4+ cells was observed mainly with DB124 and DB106 in our study. Homey and coworkers in their experiment found that treatment with oxazolone markedly induced CD69 activation marker on CD4+ lymph node cells (Homey et al., 1998). But, their results were limited to oxazolone, and needed further evaluation with a range of other chemicals.

4.2.2 B lymphocytes

In the work of Homey and coworkers (Homey et al., 1998), topical treatment with oxazolone resulted in an increase in CD45+/I-A+ lymph node cells from 1.59 to 20.89% while croton oil showed only a slight increase from 3.23 to 7.43%. In the present work, we observed a

significant increase in the CD45+/I-A+ lymph node cells in almost all the concentrations tested.

While examining whether the measurement of the percentage of CD45+/ B220+ cells could be used as an alternative or supplementary endpoint in LLNA, Gerberick and coworkers defined a B220 test: vehicle ratio cut off of 1.25 for discriminating between allergens (>1.25) and irritants (<1.25). Using B220 test: vehicle ratio of 1.25, the allergens and irritants were identified correctly in 93% of their observations (Gerberick et al., 2002). They proposed that analysis of B220+ expression may be useful in differentiating between allergen and irritant responses induced in LLNA. The increase in CD45+ cells was also reflected in the present work. The CD45 molecule, also known as “leukocyte common antigen” reached the “rational cut off” value of 1.25 proposed by Gerberick and coworkers with 3% DB124 (1.29) and 3%DB1 (1.4). However, it can not be compared directly with the work of Gerberick and coworkers, as their findings are based on a single treatment protocol and use of CBA/Ca mice.

Sikorski and coworkers observed dose-dependent changes in B lymphocytes following treatment of mice with allergens. In addition, they also demonstrated the high sensitivity of this endpoint through the investigation of different allergens. The proportion of CD45 positive cells increased more sharply following oxazolone, TNCB (1-Chloro-2,4,6-trinitrobenzol) and DNCB (1-chloro-2,4-dinitrobenzene) treatment than in mice treated with moderate allergens, such as eugenol and HCA (α - Hexylcinnamaldehyde). In contrast, the investigated irritants behaved variably with regards to increase in CD45 positive cell population. They proposed a “B / T cell ratio”, therefore an increase in B cell population and a decrease in the T cell population could be considered as a sign of an allergic reaction. In addition, they also observed a correlation between CD45 positive cells and the proportion of IgG and IgM markers on lymphocytes. This observation may provide new clues to the detection of sensitization potential (Sikorski et al., 1996). Kraal and Twisk (1984) found rise in B cells in the draining lymph nodes following treatment with oxazolone.

There might be two possible explanations regarding the preferential accumulation of B lymphocytes in the draining lymph nodes following treatment with allergens. This might be due to development of an antibody response to the hapten. Several investigators have shown that epicutaneous application of DNFB (1-fluoro-2,4-dinitrobenzene), TNCB, and oxazolone can result in formation of anti-hapten antibodies (Taylor and Iverson, 1971; Thomas et al., 1976; Askenase and Hayden, 1974; Takahashi et al., 1977; Dearman and Kimber, 1991, 1992). Other investigators suggested that anti-idiotypic antibodies may form

as a consequence of contact sensitization (Sy et al., 1979). The B cell response may also be the outcome of T cell activation. It is known that cytokines from both Th1 and Th2 cells can stimulate B lymphocytes. Neuman and coworkers observed that B cell response may result from T cell activation (Neuman et al. 1992). They noticed a dose-dependent increase in the B220+ cells as well as a decrease in the Thy 1.2, CD4, and CD8 positive cells in the popliteal lymph nodes following T cell stimulation. The increase in B220+ cells was seen because of an influx of B lymphocytes rather than increase in proliferation because few of the B220+ cells were present in the S/G_2M phase of the cell cycle.

Preferred accumulation of B lymphocytes in draining lymph nodes has also been seen in other T cell mediated immune responses. Fojtasek and coworkers studied the cell mediated immune response to Histoplasma infection in the draining lymph nodes of B6C3F1 mice following respiratory exposure (Fojtasek et al., 1993). An increase in the percentage of B lymphocytes was observed in the treated mice seven days following infection, which remained high throughout the course of infection. Constant and Wilson (1992) noticed relatively significant increase in B cells compared to T cells in draining lymph nodes following immunization with the attenuated larvae of *Schistosoma mansoni* for which the principal host defence is Th1-cell-mediated (James et al., 1986; Aitken et al., 1988; Wynn et al., 1994). This was in opposition to the Th2 response seen with the eggs of *S. mansoni* (Chensue et al., 1994). To explain this observation, they evaluated the proliferative response of the T and B lymphocytes in the draining lymph nodes. They observed relative increase in the proliferating T cells as compared to B cells. Lynch and coworkers showed that substantial accumulation of nondividing cells occurs in response to acute infection with choriomeningitis virus (Lynch et al., 1989). They found the T:B cell ratio to be decreased, indicating preferential accumulation of B lymphocytes.

4.3 General reflections on the LLNA

The present study was conducted to assess the suitability of the biphasic treatment protocol along with suitable endpoints that could be used to investigate sensitizing potential of chemicals.

4.3.1 Two-phase protocol

The emergence of contact allergy can be divided into two phases. In the induction phase, an

individual is sensitized and responds with the formation of antigen-specific memory T cells. In the second stage, the individual previously sensitized reacts on repeated exposure to the allergen with the typical signs of allergy. Thus, the most predictive tests for the assessment of allergenic effects of chemicals involve a two-phase protocol (Buehler 1965, 1985; Gad et al., 1986; Kaidbey and Klingman, 1980; Magnusson and Klingman 1969; Maurer 1980 a, b). The report on the evaluation of the LLNA by ICCVAM also supported a two-phase protocol for substances (ICCVAM, 1999).

Various irritants and phototoxic substances in the single phase protocol showed false positive results (Vohr et al., 2000; Ulrich et al., 2001). Basketter and coworkers tried to establish a threshold for distinguishing between sensitisers and irritants (Basketter et al., 1999c). The concept of using threshold for the identification of irritants and allergens in the LLNA was done on a statistical basis and does not entirely eliminate the existence of chemicals with a high potential of nonallergic lymph node hyperplasia (Dearmann et al., 1999). Ulrich and coworkers conducted experiments to classify the substances using a two-phase model (Ulrich et al., 2001). They observed that the irritation and sensitisation potentials are often linked and the interpretations of results from induction phase are often hampered by a considerable irritation potential of the test substance. In this case, they recommended the use of the two-phase approach, which was also considered to be a promising solution by the peer review panel during the ICCVAM peer review evaluation (NIH, 1999). In their study, weak allergens such as MBT, eugenol and cinnamic aldehyde showed higher lymph node activation potential in comparison to skin irritation potential, thus pointing towards allergenicity. However, irritants such as croton oil caused lymph node activation and ear irritation comparable to contact allergens with considerable skin irritation potential such as oxazolone, DNCB, DNFB and TCSA.

In a work by van Och and coworkers, mice were treated using a multi-phase protocol over a period of two months at seven days interval with allergens such as DNCB, benzocaine, and TMTD, at concentrations below the SI 3 (van Och et al., 2003). On 60th day, the proliferation of lymphocytes was measured by thymidine incorporation into the cells along with measurement of cytokine expression. The results showed that only DNCB caused a significant increase in proliferation, whereas no significant effect was observed on cytokine production. They concluded that the shorter period of exposure used in the standard LLNA protocol is sufficient enough, and longer periods of application does not have significant effects on the results.

4.3.2 Influence of the vehicle

It is necessary to take into consideration the mechanisms by which a vehicle or formulation matrix may affect the skin sensitising potential of a chemical allergen. The most important influence is to alter the skin penetration and the effectiveness with which a chemical allergen gains access to the viable epidermis. Although it is no doubt that penetration is a key factor in situation where the partition coefficient of the allergen prevents or limits access across the stratum corneum, but it is not essentially always the predominant factor. Heylings and coworkers observed that cutaneous responses by topical application to suboptimal concentrations of DNCB can be improved significantly by co-administration of the non-sensitizing surfactant sodium lauryl sulphate (Heylings et al., 1996). In situations where SLS was able to enhance the lymph node cell proliferative responses to DNCB, it failed to affect the efficiency of penetration of allergen through the skin. This shows that there are many ways in which the vehicle may affect the process of skin sensitisation.

Another effect of the vehicle might also be on the protonation state of the chemical, the protonated form having a sharply decreased ability to penetrate into the skin. Another possibility is that the vehicle influences the production of cytokines necessary for Langerhans cell migration and other cellular events at the time of contact with the chemical allergen. It might also be possible that the co-administration of SLS to enhance skin sensitisation (Kligman, 1996) increases the production of relevant proinflammatory cytokines (Grabbe and Schwarz, 1998). It is also possible that vehicle may affect the skin sensitisation by modulating the metabolic activation of a prohapten, or metabolic inactivation of the hapten itself (Basketter et al., 2001).

The influence of vehicle has been studied in animal models by various investigators. A significant influence of the vehicle on the results has been described by various workers in the LLNA (Cumberbatch et al., 1993; Warbrick et al., 1999 a,b; Basketter et al., 2001). Cumberbatch and coworkers observed that sodium lauryl sulphate (SLS) augments the skin sensitizing potential of sub-irritant concentrations of DNCB via an increase in the number of immunostimulatory dendritic cells which reach the draining lymph nodes (Cumberbatch et al., 1993). Warbrick and coworkers used acetone: olive oil (AOO), methyl ethyl ketone, DMSO, dimethylformamide, propylene glycol as vehicles to study the sensitizing potency of methylchloroisothiazolinone/ methylisothiazolinone (MCI/MI). They found that the vehicle in which MCI/MI was applied had a substantial impact on activity, with derived EC3 values ranging from 0.0049% with AOO to 0.048% with propylene glycol, while with the other vehicles EC3 values ranged from 0.0068% to 0.0076% (Warbrick et al., 1999a).

According to Kimber and coworkers, the selection of a suitable vehicle in the LLNA is one of the prerequisites for the successful assessment of the allergenic potency of a substance (Kimber and Basketter, 1992; Kimber et al., 2002). Kimber and Basketter (1992) described some lipophillic substances as suitable vehicles for the LLNA studies. These substances included acetone, acetone-olive oil (4:1), dimethylformamide (DMF), methylethylketone, propylene glycol and dimethylsulfoxide (DMSO).

Ulrich and coworkers observed that topical treatment of mice with the vehicles DMSO, AOO and DMF led to slight ear-draining lymph node activation as expressed by increased ear weights and cell counts (Ulrich et al., 2001). However, following topical administration of DAE433, a tendency to slightly decreased lymph node weight and cell counts was observed. In their experiments, DNCB induced slight but statistically significant increase in ear weight in comparison to the respective vehicle control, when DAE433 and DMSO were used as vehicles. In comparison to the untreated control, DNCB applied in AOO produced the largest changes in lymph node weight and cell counts, followed by DNCB in DAE433.

Little is known about an appropriate solubilizing agent for hydrophilic substances. It was found difficult to identify nickel as a contact allergen in animal models. No effect was observed in LLNA experiments using nickel sulphate dissolved in water at concentrations of up to 40% (Kimber and Weisenberger, 1989). Further testing with DMSO resulted in moderate lymphocyte proliferation using nickel sulphate (Kimber et al., 1990).

The suggested vehicles for the LLNA include organic solvents and organic-aqueous mixtures. However, due to its high surface tension and poor wetting qualities, water is not recommended and therefore testing aqueous soluble materials is problematic. Ryan and coworkers worked to find a water-based vehicle that possesses better skin wetting properties than water alone (Ryan et al., 2002). The selected wetting agent was the surfactant Pluronic(R) L92. Dose-response analysis was performed with dinitrobenzene sulfonic acid (DNBS) and formaldehyde formulated either in water, 1% L92, dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF). Potassium dichromate and nickel sulphate were tested in 1% L92, DMSO or DMF. While DNBS and formaldehyde produced positive responses in all the four vehicles, their relative potency varied among the vehicles. The rank ordering of potencies for both materials was, DMF > or = DMSO > 1% L92 > water. Potassium dichromate was positive in DMF, DMSO and 1% L92. The potency ranking was DMF > or = DMSO > 1% L92. Potassium dichromate in water was without activity. In DMSO, nickel sulphate produced a stimulation index (SI) >3 at only the highest level. Testing in DMF induced low levels of proliferation, but failed to produce a SI of 3 at any concentration tested.

When formulated in 1% L92, nickel sulphate caused a SI of 3 when tested at 2.5%. They concluded that LLNA, DMF and DMSO are the preferred vehicles for identification of sensitization hazard of aqueous soluble materials. However, if a test substance is not soluble in DMF or DMSO, or if higher test concentrations can be achieved in an aqueous vehicle, then 1% L92 may provide a better alternative to water.

Various workers have shown that the choice of vehicle can result in different dose-effect relationships (Ikarashi et al., 1993; Montelius et al., 1994; Lea et al., 1999; Warbrick et al., 1999). Vehicle-dependent shifts would not change the sensitizing potential of a chemical, but may cause different classifications with regards to its sensitizing potency, depending on the classification scheme used (McGarry, 2007). In the present study, DMSO was used as the vehicle and was found to be suitable for the purpose. DMSO was approved by the international committee ICCVAM, as well as from the European Organization OECD as suitable vehicle in the LLNA (ICCVAM, 1999; OECD, 2002).

4.4 LCSA

LCSA is composed of a single layer of human nondifferentiating KCs and of allogenic floating monocytes, which are cocultured in serum-free medium in presence of interleukin-4 (IL-4) and TGF-β. The loose-fit coculture matures into an allergen-sensitive system consisting of activated KCs and mobile DC-related cells (DCrc). Various chemicals such as 2,4,6-trinitrobenzenesulfonic acid (TNBS), para-phenylendiamine and 2-amino-p-cresol which are strong sensitizers and 4-aminoacetanilide, a weak sensitizer, have been tested in LCSA (Schreiner et al., 2007). The assay was found highly sensitive and reproducible in detection of the allergens tested. The LCSA could also sensitively detect metal allergens such as nickel (Ni), cobalt (Co) and zinc (Zn), the prohapten isoeugenol, and the allergens PPD (Brandowski's base) and alpha-hexyl cinnamic aldehyde (HCA) (Schreiner et al., 2008).

In the present study, the LCSA could successfully detect the allergic potential of the various textile dyes tested to a considerable extent, except Disperse blue 1. However, the results couldn't be correlated exactly to the in vivo results as the in vitro model lacks various factors which are present in an in vivo model, for e.g. the effects of skin absorption, metabolism and penetration, interactions with various other cells and the surrounding environment etc. These factors could significantly affect the results in some cases.

4.5 Effect of dye allergens

4.5.1 Disperse dyes in animal models

Dinardo and Draelos (2007) tested 33 dyes in guinea pigs using a modified Buehler and Klecak method for open epicutaneous testing. The dyes were tested at an induction concentration of 10% and challenge concentrations of 10.0%, 5.0%, and 2.5%. Nine of the 33 dyes tested produced positive allergic reactions in the guinea pig model (2-amino-4-nitrophenol, 2-amino-5-nitrophenol, acid yellow 23, acid orange 3, basic black 3, basic orange 1, disperse orange 3, solvent black 27, and solvent black 34). When eight of the nine positive dyes were retested using a 1% induction concentration, five dyes produced allergic contact dermatitis at a 1% challenge concentration (2-amino-4-nitrophenol, 2-amino-5-nitrophenol, acid yellow 23, disperse orange 3, and solvent black 34), two at a 0.5% challenge concentration (2-amino-5-nitrophenol and solvent black 34), and one at a 0.25% challenge concentration (2-amino-5-nitrophenol). DNCB at a 0.5% induction/challenge concentration was used as a positive control.

Sailstad and coworkers evaluated two dye mixtures and the individual component dyes for the potential to induce contact or pulmonary hypersensitivity (Sailstad et al., 1994). One mixture consisted of disperse blue 3 (DB3) and disperse red 11 (DR11), which are anthraquinones, and the other mixture contained DR11 and solvent red 1 (SR1), an azo dye. Contact hypersensitivity was examined using the local lymph node assay (LLNA) and a modified mouse ear swelling test (MEST). Both the MEST and the LLNA indicated that SR1 has weak contact-sensitizing potential. None of the other individual dye compounds or the two mixtures were identified as contact sensitizers by either method.

Hausen and Sawall (1989) found that testing in guinea pigs with 6 azo and anthraquinone dyes revealed that the Disperse blue 1, Disperse blue 124, and Disperse blue 3 were moderate sensitizers while Disperse red 1, Disperse yellow 3 and Disperse orange 3 were only weak ones.

Betts and coworkers measured dose-response relationships for Disperse Blue dye in LLNA (Betts et al., 2005). Their results revealed that Disperse Blue 106 had a relatively low EC3 value (0.01%), comparable to DNCB, a potent contact allergen. Ikarashi and coworkers used a sensitive mouse lymph node assay (SLNA) for detection of contact allergens (Ikarashi et al., 1996). The assay involved intradermal injection of emulsion of Freund’s complete

adjuvant along with test chemical into two sites of the abdominal skin at both sides of the ventral midline. Five days after injection, the test chemical was applied on the ears for three consecutive days. The SLNA was applied to evaluate the sensitization capacity of 20 dyes. Most of the sensitizing dyes were correctly determined as a sensitizer. Comparing the results of the SLNA with data from guinea-pig tests, the same conclusion of sensitization capacity were made in nine out of ten cases. They identified Disperse blue 1, Disperse blue 35, Disperse blue 106 and 124, Disperse orange 3, Disperse yellow 3, Disperse yellow 54, Quinoline Yellow SS, Sudan I, Sudan III, Lithol Rubine B, Brilliant Lake Red R, p-Aminophenol, p-phenylenediamine as positive, while Disperse blue 3, Disperse blue 7, Disperse red 11, Disperse red 17, and erythrosine as negative. Equivocal results were obtained for Disperse red 1 and Quinizarin. They concluded that the SLNA was sufficiently sensitive for the identification of contact allergens.

4.5.2 Disperse dyes in humans

Hausen and Sawall (1989) observed frequency of sensitization for various disperse dyes and concluded that Disperse yellow 3 is most frequently seen as a sensitizer while Disperse blue 1 and 3 are recognized very rarely. A similar observation was also determined by other authors from Europe (Cronin 1968; Cronin 1980; Berger et al., 1984; Brandle et al., 1984) and in the USA (Menezes and Hausen, 1987). These findings are also based on the fact that the yellow orange and red dyes are used more frequently than the dark ones.

In a study from Italy, Giusti and coworkers patch tested 1098 children, including 667 subjects with suspected allergic contact dermatitis and 431 patients with atopic dermatitis (AD), with seven disperse dyes: disperse blue 124 (DB124), disperse blue 106 (DB106), disperse red 1 (DR1), disperse yellow 3 (DY3), disperse orange 3 (DO3), p-aminoazobenzene (PAAB), and p-dimethylaminoazobenzene (PDAAB). Of these, 51 patients proved sensitized to disperse dyes. The most common sensitizer was DY3, followed by DO3, and DB124. Among dye-positive patients, about 12% were sensitized to disperse dyes alone and only 14% reacted to para-phenylenediamine. They concluded that in children with suspected contact sensitization, disperse dyes should be regarded as potential triggering allergens (Giusti et al., 2003).

Balato and coworkers patch tested 145 patients suspected to be sensitive to textile chemicals with an unidentified series of textile allergens (dyes, dye-related chemicals, and

resins). Twenty-three (15.9%) had positive patch test reactions to one or more dyes (Balato et al., 1990).

Hausen (1993) reported cases of allergic contact dermatitis due to leggings, containing Disperse Blue 106 and Disperse Blue 124.

Uter and coworkers found positive reactions in human patients in Germany and Austria with Disperse Blue 106 and 124, and a mixture of both (Uter et al., 1998).

In another study from Italy, Seidenari and coworkers observed Disperse blue 124 and 106 and Disperse orange 3 as the most common sensitizers among the various textile dyes tested (Seidenari et al., 2002). They also observed cross-reactivity between Disperse Blue 106 and Disperse Blue 124. Collectively, these data reveal that Disperse dyes represent a significant skin-sensitization hazard and there is need to identify their sensitisation potential.

Balato and coworkers patch tested 576 consecutive patients with the Italian Research Group on Contact and Environmental Dermatitis (GIRDCA, Italy) standard screening series and with the four Disperse dyes: Disperse Blue 124, Disperse Red 1, Disperse Orange 3, and Disperse Yellow 3 (Balato et al., 1990). These were found in the first phase to be the most common dye allergens. Nineteen (3.3%) patients were sensitive to at least one of the dyes.

In another report from Italy, Seidenari and coworkers patch tested three groups of patients (Seidenari et al., 1991). The first group included 1145 patients, 16 of whom were suspected to have textile dye dermatitis. Patch testing with the GIRDCA standard series, a textile series, and challenge tests with the suspected garments confirmed the suspected dye allergies in the 16 patients (1.4%). The second group included 861 patients who were suspected to have ACD. They were tested with the GIRDCA standard screening series supplemented with the four dispersed yes. Forty-one patients (4.8%) had positive test results. The third group included 746 patients who were suspected to have ACD, not necessarily textile related. They were tested with the GIRDCA standard screening series, the same four disperse dyes, and 12 additional dyes. Forty-three (5.8%) had positive reactions to at least one dye.

Manzini and coworkers patch tested 569 Italian patients with the GIRDCA standard screening series, a textile dye series (FIRMA, Florence, Italy), and a 17- item nondisperse dye series. Six patients (1.1%) were positive to eight textile dyes (Manzini et al., 1991).

Dooms-Goossens (1992) patch tested 3336 patients in Belgium with the European standard patch test series. Of these, 159 were tested with 15 textile dyes (Chemotechniques tandardd ye series, Malmo, Sweden) and p-aminoazobenzene. Five other patients were patch tested with p-aminoazobenzene, Disperse Yellow 3, Disperse Orange 3, and Disperse Blue 106. Twenty-eight patients had positive reactions to one of the dyes. Seventy-nine total test results were positive. The incidence yearly ranged from 0.6% to 1.1%.

Goncalo and coworkers examined the patch test results in Portuguese children younger than 14 years of age obtained by members of the Portuguese Contact Dermatitis Group between 1985 and 1989 (Goncalo et al., 1992). They found that 5 of 10,191 patients had positive patch tests to one dye, an incidence of 0.05%.

Sheretz (1992) reviewed the records of 462 patients who had undergone patch testing between 1988 and 1991. They found that two patients had positive reactions to dyes included in the Chemotechnique Diagnostic textile dye series. The incidence was therefore 0.65%.

Lazarov (2004) analysed the results from a 4-year prospective study of contact dermatitis to textiles in Israel. Six hundred and forty-four patients, referred for the investigation of contact dermatitis, and suspected of having textile allergic contact dermatitis (ACD), were studied. All patients were patch tested with the standard series (TRUE Tests), textile colour and finish series (TCFS) clothing extracts and pieces of garment in some cases. Eighty-three patients had allergic reactions to a dye and/or resin allergen. Of them, 43 had positive patch tests to the textile dye allergens, 28 to the formaldehyde and textile finish resins and 12 to allergens from both groups. The highest incidence of sensitization from the dye group allergens was due to Disperse Blue (DB) 124, DB 106 and DB 85 and from the resin group to melamine formaldehyde and ethyleneurea melamine formaldehyde and urea formaldehyde. Concomitant sensitization with allergens from the standard series included nickel sulphate, potassium dichromate, formaldehyde, rubber additives and others.

Uter and coworkers tested 1986 patients in the 31 participating centres of the Information Network of Departments of Dermatology (IVDK), all of them members of the German Contact Dermatitis Research Group, with a textile dyes series containing Disperse Blue (DB) 106 and

124, and also with a mix of both (Uter et al., 2001). 86 patients reacted positively to DB 106 and/or DB 124; and the single allergens and the mix. In contrast, cross-reaction between DB 106/124 and p-phenylenediamine and p-aminoazobenzene, respectively, was poor. Hence, DB 106/124 are important allergens deserving close monitoring.

In a study from Sweden, Ryberg and coworkers retrospectively studied 3325 patch-tested patients tested with the standard test series supplemented with Disperse blue 35, Disperse blue 106 and 124, Disperse yellow 3, Disperse orange 1 and 3, Disperse red 1 and 17 (Ryberg et al., 2006). They reported Disperse orange 1 as the most common allergen, while reactions to Disperse blue 106 and 124 were lower.

5 Summary

Title: Investigation of the sensitisation potential of various textile dyes using a biphasic mice local lymph node assay (LLNA) and an *in vitro* loose-fit coculture-based sensitisation assay (LCSA).

Contact dermatitis is one of the most common skin diseases, with a great socio-economic impact. Disperse dyes, which are suitable for dyeing synthetic fibres, are responsible for the great majority of allergic contact dermatitis (ACD) cases to textile dyes. The aim of the present study was to investigate the sensitising potential of various disperse dyes using a biphasic protocol of the local lymph node assay (LLNA) and an *in vitro* loose-fit coculture based sensitisation assay (LCSA).

In the biphasic LLNA, mice were shaved over a surface of approximately 2 cm^2 on their backs, and treated using a "sensitisation-challenge protocol". The shaved surface was treated once daily on days 1 to 3 with 50 µl of the test solution. Animals remained untreated on days 4 to 14. On days 15 to 17, mice were treated with 25 µl of the test solution on the dorsum of both ears. Mice were sacrificed on day 19 with deep CO_2 anaesthesia, the lymph nodes were prepared and various end points, such as ear thickness, ear punch weight, lymph node weight, lymph node cell count, and the proportion of various lymphocyte subpopulations were determined. The results of treated mice were compared to those of the control group treated with the vehicle alone.

The LCSA involves single layer of human non-differentiating keratinocytes and of allogenic floating monocytes which are cocultured in serum-free medium in the presence of a cytokine cocktail. The coculture develops into a system consisting of activated keratinocytes and dendritic cell-related cells. The half-maximal increase in CD86 expression on the dendritic cell-like cells (EC50 values) was used to compare the sensitising potential of tested substances.

Our results from the LLNA experiments showed that almost all of the tested textile dyes caused a significant increase in lymph node cell count and lymph node weight except Disperse orange 3. Disperse blue 124 and Disperse blue 106 showed the most pronounced increase in cell count and lymph node weight already at low concentrations of 0.003%, followed by Disperse orange 37, Disperse Red 1, Disperse Blue 35 and Disperse Blue 1.

Disperse yellow 3 showed the least increase in cell count and lymph node weight parameters among all the textile dyes tested. Based on these results, the disperse dyes could be arranged in four groups on the basis of their sensitising potency in the following decreasing order (in parenthesis: lowest concentration causing a significant increase in lymph node cell number): group 1, strong: Disperse blue 124 and Disperse blue 106 (0.003%), group 2, moderate: Disperse red 1 and Disperse blue 1 (3%), group 3, weak: Disperse orange 37 and Disperse blue 35 (10%) and group 4, very weak: Disperse yellow 3 and Disperse orange 3 (increase at 30% or no increase at 30%). We also observed an increase in ear thickness and ear-punch weight in most of the concentrations tested for various textile dyes. Many allergens cause an increase in ear parameters also, but the relative effects on lymph node parameters are more significant as compared to the ear parameters. We observed a decrease in CD4+ and CD8+ cells and an increase in CD19+, CD45+ and CD45+/1A+ cells in most of the cases, which is characteristic for allergens. The CD4+/CD69+ cells increased in only few experiments mainly with Disperse blue 124 and Disperse blue 106.

In the LCSA experiments, the textile dyes can be arranged on the basis of EC50 values in the following decreasing order of their sensitisation potency: Disperse blue 124 > Disperse yellow 3 > Disperse orange 37 > Disperse blue 106 > Disperse red 1 > Disperse blue 35 > Disperse orange 3. The results of the LCSA experiments are mostly comparable with the LLNA experiments.

In conclusion, this study shows that the biphasic LLNA protocol was proficient enough to study the sensitisation potential of tested textile dyes. The in vitro loose-fit-coculture assay also gave comparable results to LLNA, and was found to be robust enough to investigate the sensitisation potential of test substances.

Titel: „Untersuchung des sensiblisierenden Potentials von Textilfarbstoffen unter Verwendung eines zweiphasischen „local lymph node assay“ (LLNA) bei Mäusen und eines in-vitro Assays („loose-fit co-culture based sensitisation assay”, LCSA)“

Zusammenfassung:

Die Kontaktdermatitis ist eine der häufigsten Hautkrankheiten und hat eine große sozio-ökonomische Bedeutung. Verschiedene Farbstoffe, die für das Färben von synthetischen Fasern verwendet werden, sind für die große Mehrheit der Fälle von allergischer Kontaktdermatitis im Zusammenhang mit Bekleidungstextilien verantwortlich. Das Ziel der vorliegenden Arbeit war es, das Sensibilisierungspotential verschiedener Farbstoffe unter Verwendung eines zwei-phasischen Protokolls des „local lymph node assay“ (LLNA) und eines in-vitro Assays („loose-fit co-culture“) (LCSA) zu untersuchen.

Im zweiphasischen LLNA wurden Mäuse auf einer Oberfläche von ungefähr 2 cm^2 auf dem Rücken rasiert und unter Verwendung eines zweiphasischen Protokolls behandelt. Die rasierte Haut wurde einmal täglich an den Tagen 1 bis 3 mit 50 µL der Testlösung behandelt. Die Tiere blieben an den Tagen 4 bis 14 unbehandelt. An den Tagen 15 bis 17 wurden die Mäuse mit 25 µL der Testlösung auf der Rückseite beider Ohren behandelt. Am Tag 19 wurden die Mäuse mit einer CO_2-Überdosis getötet, um die Lymphknoten präparieren zu können und verschiedene Endpunkte wie Ohrstärke, Ohrstanzprobengewicht, sowie Gewicht und Zellzahl der Lymphknoten bestimmen zu können. Darüber hinaus wurden und die relativen Anteile verschiedener Lymphozytensubpopulationen durchflusszytometrisch ermittelt. Die Ergebnisse der behandelten Mäuse wurden mit denen der Vehikel-behandelten Kontrollgruppe verglichen.

Im LCSA werden nicht-differenzierte Keratinozyten vom Menschen als Monolayer und allogene Monozyten in einem serumfreien Medium in Anwesenheit eines Zytokingemisches in Kokultur gehalten. Die Kokultur entwickelt sich zu einem System von aktivierten Keratinozyten und Zellen, die dendritischen Zellen ähnlich sind. Die Halb-maximale Zunahme der Expression von CD86 auf den dendritischen Zellen (EC50 Werte) wurde verwendet, um das Sensibilisierungspotential und die Potenz der geprüften Substanzen zu vergleichen.

Die Ergebnisse der LLNA-Experimente zeigten, dass fast alle geprüften Textilfarbstoffe, ausgenommen Dispers Orange 3, einen deutlichen Anstieg der Zellzahl und des Gewichtes der Lymphknoten verursachten. Die Farbstoffe Dispers Blau 124 und Dispers Blau 106 zeigten die ausgeprägteste Zunahme dieser Parameter bereits bei niedrigen

Konzentrationen von 0,003%, gefolgt von den Farbstoffen Dispers Orange 37, Dispers Rot 1 und Dispers Blau 35. Der Farbstoff Dispers Gelb 3 verursachte unter allen geprüften Textilfarbstoffen die geringste Zunahme der Zellzahl und des Gewichtes der Lymphknoten. Basierend auf den Ergebnissen konnten die verschiedenen Färbemittel in vier Gruppen aufgeteilt werden, und zwar auf der Grundlage ihrer sensibilisierenden Potenz in abnehmender Reihenfolge (die niedrigste Konzentration, die einen signifikanten Anstieg in der Lymphknotenzellzahl verursacht, zuerst aufgezählt): Gruppe 1, stark: Dispers Blau 124 und Dispers Blau 106 (0,003%); Gruppe 2, mäßig: Dispers Rot 1 und Dispers Blau 1 (3%); Gruppe 3, schwach: Dispers Orange 37 und Dispers Blau 35 (10%) und Gruppe 4, sehr schwach: Dispers Gelb 3 und Dispers Orange 3 (Zunahme bei 30% oder keine Zunahme bei 30%). Es wurde auch eine Zunahme der Ohrstärke und des Stanzprobengewichts in den meisten Experimenten beobachtet. Viele Allergene verursachen auch eine geringe Zunahme der Ohrparameter, aber die Effekte auf die Lymphknoten sind im Vergleich damit ausgeprägter. Eine Abnahme an CD4+ und CD8+ Zellen und eine Zunahme von CD19+, CD45+ und CD45+/1A+ Zellen wurde ebenfalls in den meisten Fällen beobachtet, was charakteristisch für Allergene ist. Die CD4+/CD69+ Zellen erhöhten sich nur in wenigen Experimenten, hauptsächlich mit Dispers Blau 124 und Dispers Blau 106.

Als Resultat der in vitro-Experimente (LCSA) können die Textilfärbemittel aufgrund der EC50-Werte in abnehmender Reihenfolge ihrer sensibilisierenden Potenz folgendermaßen angeordnet werden: Dispers Blau 124 > Dispers Gelb 3 > Dispers Orange 37 > Dispers Blau 106 > Dispers Rot 1 > Dispers Blau 35 > Dispers Orange 3. Die Ergebnisse der LCSA Experimente sind demnach mit den Resultaten der LLNA Experimente größtenteils vergleichbar.

Insgesamt zeigen die Experimente, dass das zweiphasische LLNA-Protokoll geeignet war, das Sensibilisierungspotential der Textilfarbstoffe zu untersuchen. Die LCSA-Experimente ergaben vergleichbare Ergebnisse wie die LLNA-Untersuchungen. Dieser in vitro-Test scheint daher gut geeignet zu sein, um das Sensibilisierungspotential von Fremdstoffen zu untersuchen.

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7 List of Abbreviations

ACD	Allergic Contact Dermatitis
CD	Cluster of Differentiation
DB1	Disperse blue 1
DB106	Disperse blue 106
DB124	Disperse blue 124
DB35	Disperse blue 35
DCs	Dendritic cells
DCrc	Dendritic cell related cells
DMSO	Dimethyl sulfoxide
DO3	Disperse orange 3
DO37	Disperse orange 37
DR1	Disperse red 1
DTH	Delayed Type Hypersensitivity
DY3	Disperse yellow 3
EC3 value	Eliciting concentration value
EC50	Half-maximal increase in CD86 expression
FACS	Fluorescence activated cell sorter
FCA	Freund' complete adjuvant
FITC	Fluorescein isothiocyanate
FSC	Forward light scatter
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPMT	Guinea Pig Maximisation test
ICAM-1	Intercellular adhesion molecule-1
ICD	Irritant Contact Dermatitis
IFN	Interferon
Ig E/G/M	Immunoglobulin E/G/M
IL-2/4/5	Interleukin 2/4/5
KC	Keratinocyte
LCs	Langerhans cells
LCSA	loose-fit coculture based sensitisation assay
LFA-1	Leukocyte Functional Antigen-1
LLNA	Local Lymph Node Assay
LNCs	Lymph node cells
MEST	Mouse Ear Swelling Test
MHC	Major Histocompatibility Complex

NK cells	Natural killer cells
PBMCs	Peripheral Blood Mononuclear cells
PBS	Phosphate Buffered Saline
PCD	Photocontact Dermatitis
PE	phycoerythrin
PLN	Poplietal lymph node
PLNA	Poplietal lymph node assay
SI	Stimulation Index
SSC	Side-scattered light
TCR	T-cell receptors
Th1/ 2 cells	T helper 1/ 2 cells
TNF-α	Tumor necrosis factor α
UV	Ultraviolet
VLA-4	Very late antigen-4

8 List of Publications

1. **Ahuja V**, Platzek T, Fink H, Sonnenberg A, Stahlmann R (2010). Study of the sensitising potential of various textile dyes using biphasic mice local lymph node assay. *Archives of Toxicology* (Submitted)
2. **Ahuja V**, Sonnenberg A, Platzek T, Stahlmann R. Investigation of the sensitising potential of textile dyes using a biphasic protocol of the local lymph node assay. 46th Congress of the European Society of Toxicology, EUROTOX, Sept. 13-16, 2009, Dresden, Germany.

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Selbständigkeitserklärung:

Hiermit bestätige ich, Varun Ahuja, dass die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

München, den……04 May 2010…... ……Varun Ahuja……