Abstract:

Murine assays such as the mouse ear swelling test (MEST) and the local lymph node assay (LLNA) are promising alternatives to guinea pig models for the identification of contact sensitizers, yet there has been controversy over the effectiveness of these assays for the detection of weak and moderate sensitizers. Much work has been done to improve the sensitivity of the MEST. In particular, the addition of a vitamin A (VAA) enriched diet has been useful. This report further validates the use of this diet in the MEST by using a known weak sensitizer, eugenol, and a purportedly weak sensitizer, Trimelletic Anhydride (TMA). The vitamin A enriched diet improved the sensitivity of the assay such that contact sensitizing activity was identified using 20% Eugenol, a concentration which did not give positive results when vitamin A diet was omitted from the assay. There was no effect of the VAA diet on detection of TMA (25%) which was found to be a strong to moderate sensitizer. Enhanced MEST sensitivity to 20% eugenol was demonstrated in mice held on the diet for 1, 2 or 3 weeks. In contrast, 20% eugenol was not detected in the MEST when mice were maintained on the diet for 4 weeks. VAA has been reported to increase the numbers of Langerhans cells (antigen presenting cells) in the skin which could in turn enhance the cellular immune response. Since the LLNA relies on H³-thymidine incorporation by proliferating T-cells during the induction phase, we have studied the potential of the VAA diet to enhance the sensitivity of the
LLNA. Results in this report indicate that the VAA enriched diet significantly increased the assays sensitivity to eugenol and TMA (i.e. proliferation occurred) at concentrations where none was observed in mice maintained on normal chow. Previous work with formalin, glutaraldehyde and an azo dye have shown similar results. It was also demonstrated that maintenance on a vitamin A diet for 3 weeks prior to initiating the sensitization procedure was optimal. These results indicated that like the MEST, addition of a VAA diet improves the sensitivity of the LLNA a quick, objective and relatively inexpensive screen for detecting moderate and weak contact sensitizers. Using these sensitive tests, a risk assessment approach for contact sensitization has been suggested.
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**Introduction:**

Immunological reactions in the skin can be subdivided into humoral and cell-mediated responses. Humoral responses are dependent on antibody production by B-lymphocytes or plasma cells. In contrast, cell-mediated responses such as delayed type hypersensitivity (DTH) are carried out by sensitized T-lymphocytes (16).

A number of consumer products and environmental contaminants such as pesticides, textile dyes and resins, cosmetics, skin care products and cleaning products have been associated with contact sensitivity (an example of DTH), an allergic skin response to repeated chemical exposure. Thus research to develop and validate tests to assess contact sensitivity has a number of practical applications. Two such tests used to measure contact sensitivity currently under scrutiny are 1) The Local Lymph Node Assay (LLNA) and 2) The Mouse Ear Swelling Test (MEST)(20,33).

Contact sensitization occurs in two phases; an induction phase which includes antigen processing and presentation followed by an elicitation phase which is characterized by an inflammatory response upon reexposure to a particular antigen (3,10). The MEST measures contact hypersensitivity at the elicitation phase. Mice are repeatedly exposed to a chemical
for a number of consecutive days followed by a rest period which allows induction to occur. Mice are then challenged by reexposing them to the same chemical on the ears which causes an inflammatory response resulting in swelling of the ear. This change in ear thickness or edema, characteristic of contact sensitization, is measured 24 and 48 hrs after challenge. Several variations of this protocol have been tested to make it more sensitive. Of these the use of a diet supplemented with vitamin A acetate (VAA) appears to be the most useful (20,27). It has been suggested that vitamin A, through epidermal hyperplasia, increases the number of antigen presenting cells thus increasing the amount of antigen presented resulting in an increased delayed-type hypersensitivity response (20).

While the MEST measures the elicitation phase of contact hypersensitivity, the LLNA uses the induction phase to predict the contact sensitization potential of chemicals. Because contact sensitivity depends on T-cell activation by antigen presenting cells, T-cell proliferation caused by contact sensitizers was determined to be the most sensitive indicator of contact sensitivity (30). T-cell proliferation is measured by exposing animals to a chemical for 3 consecutive days after which T-cells from the lymph nodes are cultured with \(^3\text{H}\)-thymidine. Incorporation of \(^3\text{H}\)-thymidine is measured after 24 hours. The effects of vitamin A diet on this assay have not been explored. However, if Vitamin A increases the number of
antigen presenting cells (20), this would increase the amount of antigen presented to the T-cell, thus it should increase the T-cell proliferation measured by LLNA. Some preliminary work in this lab and this project investigated the potential of VAA diet to increase the sensitivity of the LLNA.

Both the MEST and LLNA have several advantages over previously validated contact sensitivity tests in that these tests use mice as opposed to guinea pigs and are less costly, and more quantitative and objective (20,31). Although both tests have been extensively validated in detecting strong sensitizers, their credibility has been questioned in detection of weak and moderate sensitizers (21,22,23). The use of Vitamin A, to make the MEST a more reliable method, has been suggested and investigated. The goals of this project were: 1) to further substantiate the observation that supplementing the diet with vitamin A prior to testing improves the sensitivity of the MEST; 2) to determine the optimum time needed to maintain mice on the diet prior to the MEST; 3) to investigate the effect of Vitamin A on the LLNA, and 4) to determine the optimum time needed on the diet to improve LLNA sensitivity.

To assess the effect of Vitamin A acetate diet on the MEST and LLNA, appropriate chemicals had to be chosen. Eugenol, a weak contact sensitizer, and Trimellitic Anhydride (TMA), a reportedly weak respiratory sensitizer (36), were chosen to test the above hypothesis. Eugenol is a component
of clove oil and has been used in pharmaceutical, cosmetic and food industries (39). TMA, a respiratory allergen, is mainly found in epoxy resins used in the manufacturing of paints, plastics, and adhesives (40). A respiratory allergen was chosen because some investigators have suggested that and have shown that several respiratory allergens also provoke weak contact sensitivity responses (36,38,47). It was important that weak sensitizers be used so that any effect of vitamin A diet on the response would be easily detected.
Literature Review:

Hypersensitivity:

Although the immune system is a defense mechanism against invasion from foreign substances, an excessive or a heightened humoral or cellular response to an antigen can lead to tissue damage. Such intense immune responses are known as hypersensitivity reactions (1). Four types (types I, II, III, IV) of hypersensitivity reactions were defined by Coombs and Gell. In the type I response, IgE binds to mast cells or basophils initiating the release of soluble mediators such as histamine resulting in bronchial asthma and/or allergy. The type II response is defined as antibody-mediated cytolysis of target cells in the lung or other tissues which have been altered to express "foreign" antigenic determinants. The type III response involves the deposition of immune complexes (between the antigen and antibody) in the lungs which activate or fix complement resulting in cell lysis. In type IV reactions, sensitized T lymphocytes induce a delayed type hypersensitivity causing inflammatory responses or direct T-cell cytotoxicity which may lead to lung injury (35). Although chemicals have been known to cause all four types of these injuries, this report will focus mainly on Type I and IV because the chemicals studied produce reactions in these
categories. The overall goal of this project was to use and improve the sensitivity of techniques previously validated to detect chemicals with the potential to cause contact sensitivity (Type IV).

Delayed-Type Hypersensitivity: (Example: Contact Hypersensitivity)

Delayed Type Hypersensitivity (DTH) reaction occurs due to an interaction between antigens and "antigen-responsive" T-cells which through cytokine production cause typical DTH skin reactions such as the Mantoux reaction to tuberculin (1,3). The Mantoux reaction is characterized by an onset within 24-48 hrs of local erythema and induration caused due to an infiltration of mononuclear phagocytes and lymphocytes (1).

Another example of a DTH reaction is allergic contact hypersensitivity (ACH). ACH is defined as an enhanced immune response to a skin allergen leading to tissue damage (4). The skin reaction to poison ivy (urushiol) is a typical example. Contact sensitization occurs when excessive antigen primed T-cells are present which can produce a delayed allergic response to that specific antigen (4). Landsteiner and Chase were the pioneers in transferring ACH to naive animals with viable lymphoid cells but not with serum from sensitized animals, making the distinction between cellular and humoral immunity (10). This set the foundation for further
 experimentation on the mechanism of ACH.

Similar to other DTH reactions, ACH reactions have two phases called the afferent and efferent phases. In the afferent or induction phase, the antigen is processed and presented to the appropriate T-helper cells by antigen presenting cells (10). It has been hypothesized that Langerhans cells (LC), dendritic type epidermal cells derived from the bone-marrow (10), carry the antigen from the skin to the draining lymph nodes (11). Evidence to support this claim include increased frequency of dendritic cells (DC) in the draining lymph nodes after exposure to skin sensitizers such as picryl chloride, oxazolone, 2,4-dinitrothiocyranobenzene (DNTB) and fluorescein isothiocyanate (FITC) (11), with increased detection of antigen on the DC's rather than T-cells in the lymph nodes 18 hours after exposure to FITC (11). Freshly isolated LC's are not immunostimulatory but become immunogenic after maturation, migration and antigen presentation in the lymph nodes (13). The hypothesized roles of LC's include capturing antigens which plays a major role in primary immune response, acting as an epidermal macrophage which takes up the antigen but under normal conditions does not have phagocytosis capabilities (12). It is thought that LC's form a reticuloepithelial trap for skin allergens and transport then through the afferent lymphatics into regional lymph nodes (14). One of the earliest events thought to occur in skin sensitization after the antigen is trapped by LCs is
the production of cytokines by keratinocytes, another cell population in the epidermis, which stimulate the migration of the LC into the lymph nodes (13). Tumor necrosis factor alpha (TNF-alpha), in addition to increasing LC viability, has been shown to be responsible for the migration of LC into lymph nodes (15). During this transport, the LC's go through a maturation phase that allows them to be better antigen presenting cells (13). The cytokines that play a major role in maturation are Interleukin 1 (IL-1) which enhances functional maturation and Granular Macrophage Colony Stimulating Factor (GM-CSF) which is responsible for maturation and viability (13). Maturation includes increased Ia antigen expression, loss of granules associated with LC and increased expression of intercellular adhesion molecule (ICAM-1) (13). These mature LCs can then act as more efficient antigen-presenting cells. It has been observed that only DCs are able to effectively carry out antigen presentation to naive T-lymphocytes while many other types of cells can present antigen to memory or primed T-cells (13), thus making DC's essential for the primary immune response (figure 1)

During antigen presentation, the antigen-carrying LC interact with CD4+ cells (T-helper cells) equipped with specific receptors for the antigen. In addition, the antigen must be presented in the presence of MHC class II molecules (Ia antigen). As mentioned above, the LC have increased
expression of this Ia (MHC II) antigen. In addition, LC have increased expression of ICAM-1, an adhesion molecule, allowing for a tight interaction between the Langerhans cell and the T-helper cells (1). The interaction of the antigen with the receptors on T-cells stimulates the Langerhans cells to produce IL-1 (3). When the neighboring T-cells are exposed to high concentrations of IL-1, a biochemical induction of RNA and protein synthesis occurs (1). These newly synthesized proteins include Interleukin 2 (IL-2) which then stimulates the cells from the G0 phase (resting) to the G1 phase (activated) of the cell cycle. IL-2 then induces the production of its own receptor on the T-cells (IL-2R), making the proliferative response optimal (3). It has been shown that there is a dose-dependent enhancement of proliferation by stimulated lymph node cells when exogenous IL-2 is added to the culture (19). This proliferative response leads to the presence of more sensitized T-cells which makes the interaction of Langerhans cell with the T-cell more likely upon re-exposure to the same antigen (3).

The second phase of contact sensitivity is the efferent phase, also referred to as elicitation phase. Elicitation occurs when the sensitized T-cells are re-exposed to the same antigen. IL-2, produced during this elicitation phase of the immune response, stimulates the production of Interferon gamma (INF-gamma). IFN-gamma amplifies the immune response by
activating cytotoxic T-cells, natural Killer cells and macrophages producing "epidermal spongiosis" or edema with cellular infiltration, characteristic endpoints of contact hypersensitivity (3).

Contact sensitivity has been associated with a number of consumer products and environmental contaminants including pesticides, textile dyes and resins, cosmetics, skin care products, and cleaning products. As a result both industry and regulatory agencies are interested in developing and validating test methods that can accurately identify contact sensitizers. Many experimental animal procedures have been developed as standard protocol to be used in testing chemicals for the potential to cause contact sensitization (16). Until recently, all of these tests have used guinea pigs as the species of choice because; 1) guinea pigs homogeneously react upon antigenic response 2) the species is tolerant of the handling associated with the procedures 3) guinea pigs have consistently given positive results for known human contact sensitizers, and 4) testing is similar to human patch testing (16,17). Some of these tests include the Draize Test (DT), Freund's Complete Adjuvant Test (FCAT), Guinea Pig Maximization Test (GPMT), Buehler Test, Optimization test, Split Adjuvant Test and Open Epicutaneous Test (OET) (16). Most of these tests administer the test material via the intradermal route to sensitize and via a combination of
intradermal or patch methods to challenge. The FCAT, GPMT and SAT use Freund’s Complete adjuvant to enhance sensitization to the test material. The adjuvant techniques have been more predictive of human results than the non-adjuvant techniques (18). All of these methods measure erythema (redness) using subjective visual rating techniques such as "+" or "-", where number of "+"'s indicate the severity of the reaction. Many variations of the above tests have been created to better estimate the sensitization potential of a chemical.

Although these methods to evaluate contact sensitivity have been extensively validated, they have several shortcomings. Several of these tests use intradermal route of administration which is far from being a realistic route of exposure. By sensitizing intradermally, the rate limiting step of penetration is avoided, thus the risk of sensitization may be overestimated (16). Other criticisms include length of the protocols, use of adjuvants, inconsistencies because of subjective evaluations especially in evaluating colored material, unclear distinction between an irritation and a sensitization response and the high costs involved in using guinea pigs (19).

Knowing these limitations, the main objective of developing new tests was to have an accurate, rapid, more quantitative, cost-effective technique that has an objective
endpoint. Two tests that have the potential to achieve these are; 1) the Mouse Ear Swelling Test (MEST) and 2) the Local Lymph Node Assay. Various versions of the mouse ear swelling assays have been performed since 1967. (20). The MEST protocol in current use follows very closely the protocol first described in 1967. Mice are topically exposed to the agent either on the back or the abdomen for a number of consecutive days, followed by a rest period which allows for induction to occur. Mice are then challenged on one ear with the agent and on the opposite ear with the vehicle. Change in edema-induced ear thickness is measured using a caliper at 24 and 48 hrs after challenge. The MEST has several advantages over the guinea pig assays which include 1) mice are less expensive and more easy to maintain, 2) the MEST gives quantitative results eliminating any bias involved in subjective evaluations, and 3) immunogenetics and skin pharmacokinetics of the mouse are better known than the guinea pig (20). Inspite of these advantages, the sensitivity and interlaboratory reproducibility of the MEST have been questioned. Using different variations of the MEST, strong sensitizers have been detected by most investigators. Detection of moderate or weak sensitizers has produced some contradictory results. For example, Gad et al. showed that the MEST was able to correctly identify 71 out of 72 test materials that varied from strong to no sensitizing potential (21), while others have found that the MEST was useful for
detecting strong sensitizers only (22,23). Because of these discrepancies, several investigators have sought opportunities to improve the MEST protocol. These ranged from methods to increase delivered dose which included changing the site of application from abdomen to the back and abrading the skin to methods to increase induction which included the use of cyclophosphamide (20).

To make the MEST a more reliable measure of contact sensitivity, the use of vitamin A has been explored by several investigators. Vitamin A has been shown to cause epidermal hyperplasia by stimulating cell production in mice, guinea pigs and in humans (20). The cell production is only seen in the stratum granulosum and stratum spinosum layers of the epidermis (24). Other properties of Vitamin A include enhanced host-vs-graft reactivity, enhanced specific anti-tumor immune response and increase in dermal delayed-type hypersensitivity reactions. In contrast, Vitamin A suppresses primary IgM responses, mitogen induced lymphocyte proliferation and phagocytosis of opsonized cells. The overall picture indicates that Vitamin A is responsible for enhancing cell-mediated immunity (20). The mechanism by which Vitamin A enhances delayed type hypersensitivity is thought to be by increasing the number of antigen presenting cells or dendritic cells. It has been shown histologically that Vitamin A fed animals had an expansion in the splenic marginal zone and the
paracortical region of the lymph nodes, with increased number of accessory cells such as dendritic cells and macrophages present (25). Effect on the number and distribution of T-cells by vitamin A have been negligible. Smith et al. (1987) showed that Vitamin A-deficient mice had a reduced delayed hypersensitivity reaction to DNFB (2,4-dinitrofluorobenzene), a contact sensitizer, and observed no significant difference between the Vitamin A+ and Vitamin A− groups in the T-lymphocyte subpopulations in thymus, lymph nodes and spleen (26). As discussed in the mechanism of contact sensitivity, antigen presentation is a key step in the induction of contact sensitivity. Vitamin A diet then can be used as a non-invasive technique to enhance induction in the MEST, thus making the assay more sensitive to weak sensitizers. Using the MEST, Maisey et al. found that mice fed with Vitamin A supplemented diet showed a DTH reaction to concentrations of oxazolone that were too low to induce a DTH reaction in mice fed the standard diet (27). They further tested 12 fragrances, 4 preservatives and 1 medicament for their sensitizing potential in a follow-up study using the Vitamin A supplemented MEST and compared the results of 8 of the compounds to guinea pig maximization test (GPMT). They found that the two tests were equally capable of predicting contact sensitizers with some variation (28). Thorne et al. tested the Vitamin A supplemented MEST using 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitrofluorobenzene (DNFB) and found that ear
swelling responses doubled in the vitamin A fed mice (20). The ability of vitamin A MEST to detect weak sensitizers was tested by Thorne et al. in a follow up study using fragrance components and complex fragrance mixtures, where the Vitamin A MEST was shown to be sensitive and predictive enough to test compounds and mixtures for contact sensitivity (29,41).

In addition to the MEST, another method for predicting contact sensitization is the Local Lymph Node Assay (LLNA). The LLNA measures contact sensitization as a function of T cell activation or more specifically T-cell proliferation in the draining lymph nodes. It is known that contact sensitivity is dependent on the activation of T-lymphocytes by topically applied contact allergens (30). Kimber et al.(30) used this initiation of the activation of T-lymphocytes as an indicator of contact sensitivity. Many markers of this lymph node activation were tested including lymph node weight, large pyroninophilic cells and T-lymphocyte proliferation of which the latter was determined to be the most sensitive indicator of contact sensitivity (30). After experimenting with many combinations, it was concluded that the LLNA was most sensitive when mice were exposed to various concentrations of test chemicals for three consecutive days. 24 hrs after the final exposure the animals were sacrificed, the draining lymph nodes were excised and a suspension of lymph node cells was prepared. These cells were then cultured with $^3$H-thymidine
for 24 hrs to measure the proliferative activity of the cells (33). An initial validation study tested 22 known contact sensitizers varying in potency. A substantial increase in lymphocyte proliferation was reported for all of these test materials with the exception of three which used water as a vehicle (31). To make this test more useful for routine testing and to avoid the need for aseptic technique, a modified LLNA was developed where instead of culturing the T-cells in vitro, proliferation was measured by injecting 3H-thymidine intravenously (33). The reliability of the LLNA was tested by performing a inter-laboratory trial using eight chemicals and comparing the results with Magnusson and Kligman guinea pig maximization test. Although some variation with the stimulation values was seen, the overall result was consistent in all the laboratories where six chemicals showed a positive response and two were reported to be negative. With the exception of one chemical, the results also correlated well with the predictions made by the guinea pig maximization test (32). The LLNA was also compared to the Buehler patch test predictions. Out of the 25 chemicals tested, "moderate and strong" sensitizers as classified by the Buehler patch test were easily detected by the LLNA while weak sensitizers were regarded as "not strong sensitizer" in the LLNA. A "not strong sensitizer" is a chemical that fails to cause a three-fold increase in \(^{3}\)HTdr incorporation. Some chemicals that gave a negative response in the Buehler patch test showed a
positive response in the LLNA, thus adding evidence which questions the sensitivity of the Buehler patch test (34). More collaborative studies using a variety of chemicals such as preservatives, perfume ingredients, surfactants, plastics/resin chemicals and oil additives were done to validate the LLNA. Again a high degree of correlation was found between the LLNA and guinea pig test data (33). Chemicals that showed the strongest response in the MEST such as DNFB and DNCB also showed a strong response in the LLNA while weak sensitizers such as eugenol and formalin that showed a variable response in the MEST also showed a positive response in the LLNA at optimal test concentrations (31). In addition to all the advantages of the MEST, the LLNA offers two more advantages. One is the fact that irritant nonsensitizing chemicals do not affect the draining lymph nodes immunologically thus avoiding the need to determine the maximal non-irritating concentration and second is the fact that the LLNA does not require the use of adjuvants (31).

To make the LLNA more sensitive, the use of IL-2 in culture has been shown to be effective where IL-2 elevated 3HTdr incorporation as compared to the controls. Mechanistically, this is not a surprise since IL-2 is a growth factor that is necessary for antigen-stimulated T-cell proliferation (31). Another option, until recently untested, to make the LLNA more sensitive is to maintain the animals on
a Vitamin A acetate diet similar to the animals used in the MEST. It is known that Vitamin A enhances contact sensitivity by causing epidermal hyperplasia which increases the number of antigen presenting cells in the epidermis. Increased antigen presentation leads to an increase in T-cell proliferation, thus causing an enhancement in subsequent steps in contact sensitivity. This increased proliferation can be used to make the LLNA assay more sensitive. Although the literature (33), suggests that such diets have not significantly increased the sensitivity of the LLNA, preliminary work in this laboratory and this report suggest that Vitamin A can enhance the sensitivity of the LLNA.

Immediate Hypersensitivity:

Immediate or respiratory hypersensitivity is a common illness seen in patients today (2). Symptoms of these patients can range from mild urticaria or seasonal rhinitis to life-threatening anaphylactic shock with bronchoconstriction (2). Most extensively studied respiratory sensitizers such as isocyanates and acid anhydrides have been known to have reactive groups which bind to the amino group of proteins in the body (lung). The immune response is induced when these chemically altered self proteins are presented to T-helper cells in the presence of the MHC II molecules. Specific cytokines are produced which stimulate the production of cytophilic antibodies (IgE) by B-lymphocytes. Upon re-
exposure to that antigen, crosslinking of the IgE molecules on the mast cells occurs which initiates the secretion of preformed mediators such as histamine, proteases, proteoglycans, and chemotactic factors, stored in the granules of mast cells. These preformed mediators allow the synthesis of membrane-derived lipid mediators such as arachidonic acid and platelet activating factor. Together these are then responsible for the physiological changes that are typical of immediate-type hypersensitivity (3). These changes include vasodilation and increased vascular permeability, bronchial and smooth muscle contraction, increased mucus production and chemotaxis for eosinophils, neutrophils, and mononuclear cells (2). The severity of these effects depends on the extent and location of mediator release. Skin, lungs, nasal membranes, tongue, and gastrointestinal tract are rich in mast cells (2).

Respiratory allergy in humans is clinically classified by time of onset. Reactions that occur within one hour of exposure are called "immediate-onset" allergic responses (IAR) and reactions occurring later than one hour are referred to as "late-onset" allergic reactions (LAR) (35). IAR are known to be IgE dependent reactions while the mechanism for LAR is uncertain. It has been suggested that high molecular antigens such as plant, animal and bacterial/fungal derived agents that have been known to cause respiratory hypersensitivity are responsible for immediate-onset reactions (4,35,43) while low
molecular weight agents which include "chemical sensitizers" such as anhydrides, isocyanates, antibiotics, metallic salts and dyes (4,5) are more likely to cause late-onset reactions (35,43). However, known respiratory sensitizers tested in several different testing schemes have provoked production of cytophilic antibody (IgE in mice; IgG in guinea pigs) adding evidence for immediate-onset rather than delayed onset (36,48).

Methods for Assessing Chemicals for Immediate-Type Hypersensitivity:

Evaluation of respiratory sensitization in humans requires a combination of skin tests which indicate the presence of type I allergy, antibody titer assays used mainly for proteins and bronchial challenge techniques (9). Much research has been done to develop new test methods in animals.

Low molecular weight agents such as acid anhydrides and isocyanates have been extensively used in developing models to test the respiratory sensitization potential. In almost all of these models, the species of choice has been guinea pigs. One such model is described by Karol et al (1985) where guinea pigs are exposed via inhalation to an allergen on days 1-5, rested until day 21 and challenged several times thereafter via inhalation. Guinea pigs are monitored for 24 hrs thus allowing the detection of immediate and late onset respiratory
hypersensitivity. Several endpoints can be assessed using this model. These include febrile reaction characteristic of late onset respiratory sensitization, specific antibodies including hypersensitivity antibodies and airway hypereactivity, a hallmark characteristic of asthma, measured as a function of increased responsiveness to inhaled histamine. This model has been validated using anhydrides and isocynates (7 chemicals) in that it has shown to appropriately distinguish between respiratory and dermal sensitizers (35).

Another model suggested by Sarlo and Clark (1992) uses a tiered approach to evaluate potential respiratory sensitizers, using both in vitro and in vivo test to investigate the allergic potential of chemicals. Level 1 of this multi-level model uses structure-activity information to see if a particular chemical has potential to bind to a carrier molecule (protein) or more importantly whether it belongs to a group of chemicals that have been known to be respiratory sensitizers. The ability to haptenate to a protein by a particular chemical is considered in level 2 of this model. If a chemical shows positive results in the in vitro tests of level 1 and 2, it is further investigated in level 3 using the guinea pig injection model where guinea pigs are subcutaneously injected twice a week for 4 weeks and reinjected with chemical after 1 week’s rest. One week after challenge, antibody testing and respiratory response to
intratracheal challenge to protein conjugate is performed. Level 4 concentrates more on guinea pig inhalation models such as the one suggested by Karol et al (1985).

Due to the recent research in understanding the mechanism behind contact and respiratory hypersensitivity, a new approach has been developed to assess the potential of chemicals to cause sensitization (35). There has been some speculation that contact and respiratory allergens cause a differential stimulation of the immune response (36). Respiratory sensitizers are thought to produce IgE, antibody responsible for immediate hypersensitivity, and IgG2b antibodies while contact sensitizers are thought to inhibit IgE and stimulate production of IgG2a (37). It has been suggested that this differential production of antibodies is due to the fact that contact and respiratory allergens stimulate different subsets of T-helper cells. (34,35). Many in vitro and some in vivo studies have developed models suggesting that different subsets of T-helper cells exist and they regulate different sets of immune and inflammatory responses (42). These findings have been applied to respiratory and contact allergens. Respiratory allergens are said to selectively stimulate Th2 cells which when activated produce interleukin 4 (IL-4), thus stimulating the production of IgE (34,35). This is called selective stimulation rather than exclusive stimulation because respiratory allergens can also cause weak to moderate contact sensitization (38). Th1
cells, thought to be activated by contact sensitizers, produce IFN-γ which inhibits the production of IgE. These findings have led to the development of The Mouse IgE Test or analysis of serum IgE (35). When mice were topically exposed to either TMA (Trimellitic anhydride), a respiratory sensitizer, and DNBC (2,4-Dinitrochlorobenzene), a contact sensitizer, serum IgE levels significantly increased only in the TMA exposed animals while elements of contact hypersensitivity were seen in both TMA and DNBC exposed mice (37).
Materials and Methods:

Chemicals: Eugenol (Aldrich Chemical Company, Inc) solutions were made using 99% Eugenol diluted to the appropriate concentrations in spectrophotometric-grade acetone (Mallinckrodt, Paris, KY, U.S.A). 97% 1,2,4-Benzenetricarboxylic anhydride, otherwise called trimellitic anhydride (TMA) (Aldrich Chemical Company, Inc) was used to make TMA dilutions using acetone. The upper most test concentrations were chosen according to the solubility of the chemical in the vehicle (acetone) used.

Mice: Four-week-old female Balb/c mice (Charles River, Raleigh, NC) were group housed in suspended polycarbonate cages with bedding of pine shavings in an environmentally controlled, American Association for Accreditation of Laboratory Animal Care-accredited, vivarium. Randomly selected animals tested serologically on arrival and sentinel mice monitored serologically throughout the study were free of Sendai virus, pneumonia virus of mice, mouse hepatitis virus, other murine viruses, and mycoplasma. Mice were also monitored for and found to be free of ectoparasites and endoparasites. Mice were either placed on a standard diet (Purina Rodent Lab Chow, St. Louis, MO) or on standard diet
supplemented with 0.477 g/kg vitamin A acetate (15 times the amount found in standard diet) in the form of stable gelatinized beadlets (ICN Biochemical, Cleveland, OH) for a time period determined to be appropriate for the experiment. Diets were stored at 4 C and used within 6 months to avoid loss of vitamin A activity. Testing procedures were carried out when mice were between the ages of 8-12 weeks.

**Mouse Ear Swelling Test (MEST):** The mouse ear swelling test was performed as previously described by Sailstad et al (1993). Mice were anesthetized with metofane and the backs were shaved using styling clippers (SLII, Andis Company, Racine, WI) the day prior to sensitization. Mice were sensitized on the shaved area with 100 ul of the test agent or vehicle for 2 consecutive days. On day 5, mice were challenged with acetone (vehicle) on one ear and test agent on the other. Treated and vehicle ear thickness measurement were taken prior to challenge and 24 and 48 h after the challenge using an Oditest D1000 hand-held caliper with reduced spring tension (approximately 36 grams of force) (The Dyer Co., Lancaster, PA). Three measurements were taken at the apex of each ear. Ear measurements were alternated between right and left to reduce compression artifacts. Measurements were performed at approximately the same time and under the same conditions each day.

**Local Lymph Node Assay (LLNA):** Twenty-five microliters of
the agent solution (TMA or Eugenol) or acetone were applied to the ears of Balb/c mice for 3 days. On the 4th day, mice were sacrificed by cervical dislocation and the auricular lymph nodes were removed. Asseptic techniques were used throughout the assay. Due to the limited number of cells in each node, pools were made using the nodes of 3 mice. Three pools were used for each agent or acetone-control group. Pooled nodes were homogenized to a single cell suspension using glass tissue grinders. Cell mixtures of 1.2 * 10^6 cells were added to wells of a 96-well microtiter plate in an RPMI-1640 media containing L-glutamine, 25Mm Hepes, 10% fetal bovine serum and 2% penicillin-streptomycin (Gibco Laboratories, Grand Island, NY). Tritiated thymidine (2uCi/well) (New Dupont, Boston, MA) was added to each well and incubated for 24 hours at 37 C and 5% CO₂. The cells from each well were harvested to filter disks using a Skatron cell harvester (Skatron Instruments, AS, Norway). Incorporation of ³H was determined using a Beta Scintillation Counter (Model 2500 TR, Packard Instrument Company, Meridan, CT).
Statistical Analysis:

MEST: For each test animal, triplicate measurements of ear thickness were averaged and the ear thickness of the vehicle-challenged ear was subtracted from the thickness of the treated ear. To adjust for non-treatment related differences, the pre-challenge (0 h) value was subtracted from the post-challenge (24 and 48 h) values. A repeated measures multivariate analysis of variance (MANOVA) was applied to the adjusted measurements comparing the responses of unsensitized and sensitized mice at the two post-challenge times.

LLNA: Experimental and control groups each contained nine mice. Groups were divided into three subgroups or "pools" of three mice apiece. Auricular lymph nodes from each pooled subgroup were processed as described in the methods section. The counts per minute (CPM) were obtained from triplicates of each pool. The average of these triplicate readings was used as input for the statistical evaluation using an analysis of variance. Non-treatment differences between experiments were adjusted for in the analysis by comparing common groups and noting the differences and adjusting the other groups for those differences. To analyze the vitamin A timecourse data using 40% eugenol (Figure 6b), the control values for each
group were averaged. Each treated CPM value was adjusted by the average of its corresponding control group. This adjusted CPM value served as the dependent variable for the one-way analysis of variance performed on this data. Significance levels were adjusted for multiple comparisons using a modified Bonferroni correction.
Results:
Effect of Vitamin A on the Mouse Ear Swelling Test (MEST):
Response to Eugenol:

Mice were fed either the vitamin A supplemented diet for 3 weeks or standard diet (0 weeks on Vitamin A) and the effects of Eugenol were assessed using the MEST. A significant change in ear thickness caused by 20% eugenol was only observed in mice maintained on the vitamin A supplemented diet. No statistically significant increase in ear thickness was observed in mice fed the standard diet. These results were independent of whether the measurements were taken at 24 or 48 hours, but the response increased after 48 hrs (Figure 2a and 2b).

To determine the minimum time required on vitamin A in order to detect contact sensitivity response as a result of 20% eugenol exposure, animals were maintained on the vitamin A supplemented diet for 1, 2 and 4 weeks before the MEST was performed. A statistically significant increase in ear thickness was seen in animals on the supplemented diet for 1 and 2 weeks of Vitamin A at 24 hrs, but detectable change in ear thickness was not seen in animal fed vitamin A diet for 4 weeks. Similar trend in responses were seen at 48 hrs but the
overall change in ear thickness at all time points increased (Figure 3a and 3b).

Response to TMA:

Mice fed either the standard diet or vitamin A supplemented diet were exposed to 25% TMA as described in the MEST protocol. Contact sensitization by TMA was detected in both the vitamin A and the non-vitamin A fed groups 24 hours after challenge (Fig 4a). The response decreased 48 hrs after challenge but increase in ear thickness was still statistically significant in both the TMA-treated groups (0 and 3 weeks) as compared to the controls (Fig 4b).

Effect of Vitamin A on the Local Lymph Node Assay (LLNA)

Response to Eugenol:

Two concentrations (10 and 20%) of eugenol were initially tested in the LLNA using mice on the standard diet or on the diet supplemented with 0.477g/kg Vitamin A for 3 weeks. Control mice in each diet group were treated with acetone (vehicle). Results show no increase in proliferation in the local lymph node as a result of treatment with 10% eugenol (Figure 5). This was true whether mice were fed the standard diet or the Vitamin A acetate supplemented diet. In contrast, treatment with 20% eugenol caused increased proliferation in the local lymph node in mice fed the vitamin A supplemented diet but not of mice fed the standard diet (Figure 5).
Since the Vitamin A supplemented diet appeared to increase the sensitivity of the LLNA, experiments were done to determine the amount of time that mice were required to be on Vitamin A to give an optimum response in the LLNA. Using 40% eugenol, the LLNA was performed on mice fed Vitamin A supplemented diet for 0, 1, 2, 3, or 4 weeks. Eugenol treated animals showed a significantly higher response in all of the weeks tested (Figure 6a), but after further analysis of the data (discussed in Statistical Analysis section), the optimum response was seen in animals fed the Vitamin A supplemented diet for 3 or 4 weeks (Figure 6b).

Response to TMA:

Various concentrations of another reportedly weak contact sensitizer, Trimellitic anhydride, (TMA) were tested. 6, 3, 1.5, 0.5 % TMA was tested in the LLNA using mice fed the standard diet. Significant cell proliferation was seen in animals exposed to 1.5, 3, and 6% TMA as compared to the controls (animals exposed to acetone) but the difference was not statistically significant at 0.5% TMA (Figure 7a).

A similar assay was performed using mice fed Vitamin A supplemented diet. TMA concentrations of 0.5, 1.5, 12.5, 25, 41% were tested in the LLNA. High T-cell proliferation as indicated by \(^3\)H-incorporation was detected at 12.5, 25 and 41% as compared to the control animals. Statistically significant difference in cell proliferation was also seen in animals
treated with concentrations as low as 0.5% TMA (Figure 7b) which was in contrast to 0.5% TMA treated animals fed control diet (Figure 7a) in which increased cell proliferation was not detected.

Table 1: Summary of Results:

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>CONCENTRATION (%W/V)</th>
<th>LLNA + VIT A</th>
<th>LLNA - VIT A</th>
<th>MEST + VIT A</th>
<th>MEST - VIT A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eugenol</td>
<td>40</td>
<td>+</td>
<td>+</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>TMA</td>
<td>41.5</td>
<td>+</td>
<td>.</td>
<td>.</td>
<td>[A .</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>+</td>
<td>.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>+</td>
<td>.</td>
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<td></td>
<td>6.0</td>
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<td>+</td>
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<td></td>
<td>3.0</td>
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<td>+</td>
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<td>.</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td></td>
<td>0.5</td>
<td>+</td>
<td>-</td>
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</tr>
</tbody>
</table>

+ could be detected (p<.05)
- cannot be detected (p>.05)
. not tested
Discussion:

This study examines the role of vitamin A enriched diet in enhancing the sensitivity of two murine contact sensitivity assays, the Mouse Ear Swelling Test (MEST) and the Local Lymph Node Assay (LLNA). Specifically, the sensitivity of both assays was tested using two contact sensitizers, Eugenol and Trimelletic anhydride (28,36). The MEST and the LLNA have had repeated success in detecting strong to moderate contact sensitizers (21,31,32), but have been criticized for their inconsistency in detecting weak sensitizers (21,22,23). With the addition of a vitamin A enriched diet, the MEST has been shown to detect weak sensitizers and low concentrations of moderate sensitizers (20,28,41). Similar results in the MEST were obtained in this study where 20% eugenol showed a delayed-type hypersensitivity (DTH) reaction only in mice fed the vitamin A diet while no significant increase in ear thickness, characteristic of a DTH reaction, was seen in animals fed the standard diet (Figure 2a and 2b). While vitamin A is crucial in detecting weak DTH responses such as the one seen with eugenol, it has no effect when DTH responses are high. An example in this report would be TMA at 25% w/v (Figure 4a and 4b), which was detected in the MEST regardless of the diet used. Previous work in this laboratory with
other sensitizers such as formalin and an azo dye have produced similar results where concentrations too low to elicit an DTH response in mice fed the standard diet have produced a DTH reaction in mice fed the Vitamin A enriched diet. This report not only further extended the hypothesis that vitamin A enriched diet enhances the ability of the MEST, but also determined that maintaining mice on Vitamin A diet for as little as 7 days (1 week) increases the sensitivity of the MEST (figure 3a) in detecting 20% eugenol. Prior to this finding, mice were maintained for an average of 21 days on vitamin A, making it relatively more expensive. Interestingly, a DTH response to eugenol was not detected when mice were fed the vitamin A diet for 4 weeks. A similar reduced effect was observed when Thorne et al (1987) administered high doses of certain diisocyanates. It has been proposed that this reduced response at high doses results from down-regulation of the immune system (47). It could be speculated that high levels of Vitamin A may cause a similar down-regulation leading to a decrease in the DTH response.

While the effect of VAA diet has been previously studied in the MEST, it had not been explored in the LLNA. Various investigators have tried to increase the sensitivity of the LLNA by varying the preliminary exposure site, exposure length and exposure regimen (i.e. three smaller doses are more effective than one strong dose) (44). Gerberick et al (1992) tried to increase the sensitivity of the LLNA by increasing
the number of test applications from three to four. Even with this modification, two weak contact sensitizers went undetected in the LLNA. In this study, we tried to increase the sensitivity of the LLNA using VAA diet. We found that detection of 20% eugenol in the LLNA was only possible in mice fed the vitamin A enriched diet (Figure 5). Similar results were obtained with 0.5% TMA which also showed significant proliferation only in the Vitamin A diet fed mice (Figure 7a and 7b). Vitamin A has been shown to cause epidermal hyperplasia (20) which may lead to an increase in the number of antigen presenting cells in the epidermis (25). Increased antigen presentation may lead to an increase in T-cell proliferation. Since the LLNA measures T-cell proliferation by radiolabeled thymidine incorporation, this could explain the increase in sensitivity of the LLNA by Vitamin A enriched diet. Unlike the MEST, obtaining an optimal eugenol response in the LLNA requires at least three weeks of vitamin A supplemented diet prior to sensitization (Figure 6a and 6b).

Many investigators have developed methods to compare potencies of various contact sensitizers. Thorne et al (1987) express potencies as the SD50 or dose required in the MEST to sensitize 50% of the animals in the group. A larger SD50 would imply a weaker contact sensitizers. The LLNA has also been used to rank contact sensitizers. Gerberick et al (1992) apply a crude method where they arbitrarily rank a chemical as
"moderate to strong" if it demonstrated a >30 fold increase in [3H]TdR incorporation, "weak to moderate" if it demonstrated a >2-30 fold increase in [3H]TdR incorporation and chemicals that failed to shown a greater than 2 fold response were not considered to be contact sensitizers. Kimber et al (1991) compared [3H]TdR of TMA to 2,4-dinitrochlorobenzene (DNCB) and reported TMA as a "definite" but "relatively weak" sensitizer. We did a similar comparison with TMA and Eugenol. 20% Eugenol, when tested with a vitamin A diet, gives a comparable proliferative response to 1.5% TMA in the LLNA, thus implying that TMA is a much stronger contact sensitizer than eugenol which is categorized as a "weak" sensitizer by Gerberick et al (1992). The point being that definitions of strong and weak sensitizers depend very much on the investigators perspective and the positive controls that are used. However, to obtain an overall list of contact sensitizers in terms of their potencies will require extensive research and data gathering. When assigning potencies, exposure must also be taken into consideration. For example, it takes 50% TMA to produce the same proliferation response in the LLNA as 1% DNCB (36). However in an occupational setting, it may be more common to be exposed to 50% TMA than 1% DNCB, thus increasing the risk of contact sensitization from TMA over DNCB.

Increasing the sensitivity of both the LLNA and the MEST has several implications in contact sensitization risk assessment. Gerberick et al (1992) have proposed a scheme
invoking a combination of the Local lymph node assay with guinea pig sensitization tests to assess risk of contact sensitization from a particular chemical. By using the Vitamin A-LLNA and substituting the MEST for guinea pig tests, assessing risk may be more accurate, less expensive and more objective.
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FIGURES
**Figure 1**: A schematic representation of the induction phase of contact hypersensitivity.
**Figure 2a:** Eugenol response in the MEST using mice fed either Vitamin A supplemented diet for 3 weeks or the standard diet (0 weeks). Mice were sensitized and challenged with 20% eugenol. Results are expressed as the thickness of the ear challenged with chemical minus the thickness of ear "challenged" with vehicle 24 hours after challenge. Values are adjusted for pretreated differences. *Statistically different from mice "sensitized" with vehicle (p<.05).
Figure 2b: Eugenol response in the MEST using mice fed either vitamin A supplemented diet for 3 weeks or the standard diet (0 weeks). Mice were sensitized and challenged with 20% eugenol. Results are expressed as the thickness of the ear challenged with chemical minus the thickness of ear “challenged” with vehicle 48 hours after challenge. Values are adjusted for pretreated differences. *Statistically different from mice “sensitized” with vehicle (p<.05)
EUGENOL (20%)
ACETONE

TIME ON VITAMIN A (WEEKS)

Figure 3a: Eugenol response in the MEST using mice fed vitamin A supplemented diet for 4, 2, and 1 weeks. Mice were sensitized and challenged with 20% eugenol. Results are expressed as the thickness of the ear challenged with chemical minus the thickness of ear “challenged” with vehicle 24 hours after challenge. Values are adjusted for pretreated differences. *Statistically different from mice “senstized” with vehicle (p<.05).
Figures 3b: Eugenol response in the MEST using mice fed vitamin A supplemented diet for 4, 2, and 1 weeks. Mice were sensitized and challenged with 20% eugenol. Results are expressed as the thickness of the ear challenged with chemical minus the thickness of ear "challenged" with vehicle 48 hours after challenge. Values are adjusted for pretreated differences. *Statistically different from mice "sensitized" with vehicle (p<.05).
**Figure 4a:** TMA response in the MEST using mice fed either vitamin A supplemented diet for 3 weeks or the standard diet (0 weeks). Mice were sensitized and challenged with 25% TMA. Results are expressed as the thickness of the ear challenged with chemical minus the thickness of ear "challenged" with vehicle for 24 hours after challenge. Values are adjusted for pretreated differences. *Statistically different from mice "sensitized" with vehicle (p<.05).
Figure 4b: TMA response in the MEST using mice fed either vitamin A supplemented diet for 3 weeks or the standard diet (0 weeks). Mice were sensitized and challenged with 25% TMA. Results are expressed as the thickness of the ear challenged with chemical minus the thickness of ear "challenged" with vehicle 48 hours after challenge. Values are adjusted for pretreated differences. *Statistically different from mice "sensitized" with vehicle (p<.05).
**Figure 5:** Response to 10 and 20% eugenol in the LLNA using mice fed either vitamin A supplemented diet for 3 weeks (Vit A+) or the standard diet (Vit A-). Each value represents the mean and standard error for 3 pools of nodes extracted from 3 mice (2 nodes/mice). Data is expressed as counts per minute (CPM). *Statistically greater proliferation from mice fed the corresponding diet and sensitized with acetone (p<.05).*
**Figure 6a:** Response to 40% eugenol in the LLNA using mice fed the vitamin A supplemented diet for 0, 1, 2, 3, and 4 weeks. Each value represents the mean and standard error for 6 pools of nodes extracted from 6 mice collected over 2 experiments. Adjustments were made to compensate the differences between two experiments. *Statistically greater proliferation from mice fed vitamin A diet for corresponding weeks and sensitized with acetone (p<.05).
Figure 6b: Response to 40% eugenol in the LLNA using mice fed the vitamin A supplemented diet for 0, 1, 2, 3, and 4 weeks. Data is presented as CPM of treated group minus the average CPM of control group corresponding to that week. Each value represents the mean and standard error of the adjusted CPM. *Statistically different from mice fed the standard diet (0 weeks) and treated with eugenol (p<.05).
**Figure 7a:** TMA response in LLNA using mice fed the standard diet. Mice were sensitized either with acetone (control) or 0.5, 1.5, 3.0, 6.0 w/v TMA. Each value represents the mean and standard error for 3 pools of nodes extracted from 3 mice (2 nodes/mice). Statistical adjustments were made to adjust for differences between two experiments.

*Statistically different from the corresponding acetone control (p<.05).
Figure 7b: TMA response in LLNA using mice fed the vitamin A supplemented diet for 3 weeks prior to sensitization. Mice were sensitized either with acetone (control) or 0.5, 1.5, 12.5, 25, 41 % w/v TMA. Each value represents the mean and standard error for 3 pools of nodes extracted from 3 mice (2 nodes/mice). Statistical adjustment were made to adjust for differences between two experiments. *Statistically different from the corresponding acetone control (p<.05).