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Evaluation of Allergic Hypersensitivity to 2,4-D, Malathion, and Captafol in Mice

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EVALUATION OF ALLERGIC HYPERSENSITIVITY TO 2,4-D, MALATHION, AND CAPTAFOL IN MICE

by

Janette R. Cushman

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Janette R. Cushman

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LIST OF ABBREVIATIONS

Abbreviation	Definition
BSA	bovine serum albumin
CPM	counts per minute
2,4-D	2,4-dichlorophenoxyacetic acid
DNFB	2,4-dinitro-5-fluorobenzene
DNP	2,4-dinitrophenyl
DTH	delayed-type hypersensitivity
ECF-A	eosinophil chemotactic factor
EDC	1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride
EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydro-quinoline
ELISA	enzyme-linked immunosorbent assay
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
KLH	keyhole limpet hemocyanin
MHC	major histocompatability complex
MMA	malathion metabolite anhydride, anhydride of [(dimethoxyphosphinothioyl)thio]butanedioic acid
N.b.	Nippostrongylus brasiliensis
PBS	phosphate buffered saline
PCA	passive cutaneous anaphylaxis
PIMS	Pesticide Incident Monitoring Service of the U.S. Environmental Protection Agency

PRAST	paper radioallergosorbent test
RAST	radioallergosorbent test
SRS-A	slow-reacting substance of anaphylaxis
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
T_{aux}	auxiliary T lymphocytes that facilitate the function of T_s
T_{DTH}	T lymphocytes that produce the delayed-type hypersensitivity response, also called T_{DH}
T_s	suppressor T lymphocytes

ABSTRACT

Evaluation of Allergic Hypersensitivity to

2,4-D, Malathion, and Captafol in Mice

by

Janette R. Cushman, Doctor of Philosophy

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Major Professor: Joseph C. Street
Department: Interdepartmental Program in Toxicology

2,4-D-specific IgE antibodies were detected in serum of BALB/c mice using the rat passive cutaneous anaphylaxis (PCA) test following the second intraperitoneal immunization with 1, 10, or 100 ug of 2,4-D-keyhole limpet hemocyanin (KLH) conjugate administered with aluminum hydroxide adjuvant. The groups that received 1 ug of 2,4-D conjugate had the highest antibody titers (geometric mean of 60). A paper radioallergosorbent test (PRAST) was developed for determination of 2,4-D-specific IgE. The PRAST was equally as sensitive as and showed a positive correlation with the PCA assay.

The anhydride of the diacid metabolite of malathion (MMA) coupled to KLH elicited MMA-specific IgE following secondary immunization with 10 and 100 ug and tertiary immunizations with 1, 10, and 100 ug of conjugate. The highest PCA titers (geometric means of 208 and 195) were found after three immunizations with 10 or 100 ug of conjugate, respectively. A PRAST for MMA-specific

IgE was developed and yielded results equivalent to those obtained using the PCA procedure.

Concurrently with these studies, dinitrophenyl-specific IgE elicited with 1 ug of dinitrophenyl-KLH conjugate was measured by the PCA test at all intervals examined.

2,4-D and malathion applied epicutaneously on two days or over four weeks failed to elicit delayed-type hypersensitivity (DTH). Captafol produced DTH responses at both dose levels tested. Following two applications, ear thickness, incorporation of 5-[^{125}I]iodo-2'deoxyuridine-labelled cells, and histology of ears indicated swelling and cellular infiltration. Multiple sensitizations over the period of a month also produced DTH as indicated by increases in ear thicknesses. Mice pretreated with cyclophosphamide produced larger responses 24 hours post-challenge but equivalent responses at 48 hours as compared to mice pretreated with saline. Sensitization with a known sensitizer, dinitrofluorobenzene, also elicited DTH.

Neither 2,4-D- nor MMA-specific IgE antibodies were detected in serum during the four week epicutaneous sensitization period. Low titers of dinitrophenyl-specific IgE were elicited in mice treated with dinitrofluorobenzene.

(129 pages)

INTRODUCTION

In recent years, the possible effects of chemicals on the immune system of exposed organism has begun to concern scientists in industry and federal agencies charged with determining potential hazards associated with the manufacturing and use of chemicals. Alterations in the normal functioning of the immune system may be expressed as a specific response to the chemical itself, a suppression or enhancement of a normal function such as suppressed resistance to an infectious agent, or an inappropriate response to a body constituent such as production of antibodies against DNA or RNA. The elicitation of allergic responses specific to and triggered by environmental chemicals is the subject of this research.

As a class of chemicals, pesticides are unique and particularly suspect due to their intended use as agents to kill selected organisms designated as "pests". Exposure to humans occurs during and after the deliberate release of the pesticides into the environment as well as during their manufacture. Effects on the immune systems of exposed humans are reported relatively frequently as compared to reports of acute or chronic toxicity. In particular, allergic responses to pesticides have been observed (Street, 1981). Case studies frequently have contained descriptions of dermatitis characterized by erythema, edema, and eczema which in many instances have been confirmed by patch

testing with the pesticides in question. Upon occasion, symptoms of chemical-related asthma, atopy, or anaphylaxis also have been reported.

Although allergic reactions to pesticides may only occur in a small proportion of the exposed population, these responses may cause great discomfort in the susceptible individuals which can lead to loss of work days or a forced change in occupation. In cases of anaphylaxis, the life of the individual may even be threatened.

Allergic hypersensitivity to pesticides has received little attention from researchers. In general, studies have been limited to the investigation of contact hypersensitivity in guinea pigs and frequently the results of these studies have not been published because of industrial proprietary interests in the pesticides under study. Therefore, this project was undertaken to assess allergic hypersensitivity to three common pesticides which have been reported to cause allergic hypersensitivity in humans. These studies were conducted using an inbred strain of mice, the BALB/c strain, as the animal model.

REVIEW OF THE LITERATURE

Allergic Hypersensitivity

An allergic response is an immunologically mediated response to an environmental antigen in which pathological reactions occur on reexposure to that antigen or a structurally similar substance. Coombs and Gell (1975) have classified allergic diseases into four types based on the nature of the immunological reaction:

Type 1: Antibodies, primarily immunoglobulin E (IgE), bind to mast cells and basophils and react with antigen causing release of chemical mediators such as histamine, slow-reacting substance of anaphylaxis (SRS-A), and eosinophil chemotactic factor (ECF-A). Diseases mediated by a Type 1 mechanism include atopy, anaphylaxis, urticaria, and angioedema.

Type II: Cytotoxicity is due to the reaction of immunoglobulins G or M (IgG or IgM) with cell-bound antigen which activates complement and causes cell lysis.

Type III: Antigen-antibody (IgG or IgM) complexes form in the circulation, fix complement, and are deposited in vessel walls where they generate neutrophil chemotactic factors and cause local tissue inflammation. Examples are serum sickness and the Arthus reaction.

Type IV: T lymphocytes previously sensitized by contact with the antigen produce lymphokines upon reencountering the antigen,

resulting in inflammation. This mechanism is responsible for delayed-type hypersensitivity which includes allergic contact sensitivity and tuberculin-type hypersensitivity.

Allergic hypersensitivity to environmental chemicals is usually of Type I (IgE antibody-mediated hypersensitivity) or Type IV (delayed-type hypersensitivity). These two types are discussed below.

Allergic reactions mediated by IgE antibodies

Description. Allergic reactions in humans such as generalized or local anaphylaxis and atopy are mediated primarily by IgE, with cytotropic IgG playing a lesser role. IgE bound with high affinity to specific membrane receptors on basophils and mast cells combine with antigen, initiating the release of vasoactive amines and other chemicals which produce the symptoms of hypersensitivity (Frick, 1980). Histamine causes smooth muscle contractions of human bronchioles and small blood vessels, increased permeability of capillaries, and increased secretion by nasal and bronchial mucous glands. SRS-A causes constriction of smooth muscle and recently has been identified as a leukotriene compound which is a metabolite of arachidonic acid (Jakschik et al., 1977). ECF-A also is released and results in the accumulation of eosinophils in the area of inflammation. Smooth muscle contraction, increased vascular permeability, and increased mucous secretion are also caused by bradykinin from mast cells. Other species release serotonin and platelet-aggregating factor in

addition. The elicitation of symptoms in the exposed skin, respiratory system, or gastrointestinal tract occurs within minutes following exposure of a sensitized person to the antigen, which lead early observers to name this type of reaction immediate hypersensitivity.

The identification of IgE as the entity responsible for immediate hypersensitivity was made by Ishizaka and coworkers (Ishizaka et al., 1966a, 1966b; Ishizaka and Ishizaka, 1967). IgE has a molecular weight of approximately 190,000 daltons, slightly larger than the monomers of the other immunoglobulins (Johansson and Bennich, 1967). Two light chains (both either k or l) and two heavy chains (type e) comprise the molecule, as shown in Figure 1. Side chains of oligosaccharides are attached to the main amino acid chains, and constitute 12% of the molecular weight. Both light and heavy chains have variable and constant regions. The variable regions are found in the $F(ab)_2$ fragment resulting from cleavage by papain and are responsible for the antibody's ability to bind a specific antigen (Ishizaka et al., 1970b). The constant regions do not differ between IgEs with different antigen specification. The C_3 and C_4 regions in the Fc (crystallizable) fragment from papain cleavage are involved in binding of the IgE molecule to the receptor on mast cells and basophils (Stanworth et al., 1968; Ishizaka et al., 1970a). Additional information about the structural and physical properties of IgE can be found in the review by Dorrington and Bennich (1978).

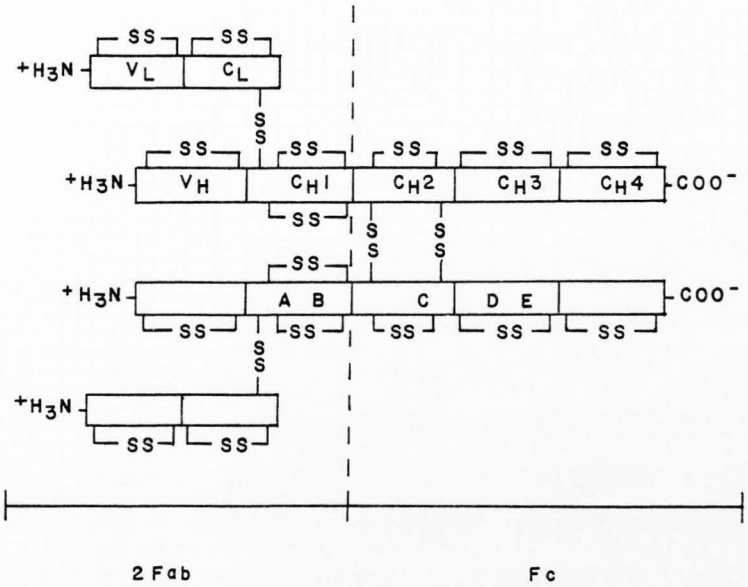

Figure 1. Schematic model of IgE showing the fragments obtained upon cleavage with papain (Fab and Fc), the variable (V) and constant (C) regions of the light (L) and heavy (H) chains and the sites of carbohydrate attachment (A, B, C, etc.) (modified from Dorrington and Bennich, 1978).

IgE is normally found in the serum at the lowest level of any immunoglobulin, an average of about 90 to 220 ng per ml for total IgE in adults (Sears et al., 1980). Elevated total IgE or IgE specific for an antigen has been reported in patients with a variety of allergic diseases, including asthma (Johansson, 1967; Wide et al., 1967), allergies to ragweed, grass pollen, dust, mites, and insect stings (Aas and Johansson, 1971; Berg et al., 1971; Norman et al., 1973; Chapman and Platts-Mills, 1978; Sobotka et al., 1978), dermatitides of various forms (Schur et al., 1974); O'Loughlin et al., 1977; Wuthrich, 1978), and respiratory hypersensitivity to toluene diisocyanate (Karol et al., 1978), phthalic anhydride (Maccia et al., 1976), trimellitic anhydride (Zeiss et al., 1977), and coffee beans (Karr et al., 1978).

Infestation with helminth parasites also results in increased serum IgE (Jarrett, 1978). In a recent position paper, Capron and Dessaint (1981) reviewed the evidence that IgE produced during helminth infestations interacts with eosinophils and macrophages to activate them directly by binding to these cells or through binding of the IgE to mast cells. This role of IgE as a regulator of cell activity extends the functions of IgE beyond that of simply a mediator of pathologic reactions of immediate hypersensitivity.

Values for total IgE show a great variation in both normal subjects and in asthmatic and allergic patients. The variability may be due to a combination of the influence of a family history

of allergy (Sears et al., 1980), the selection of the normal reference group, differences in technique, time of year, and severity of disease (Foucard and Johansson, 1976). A much better correlation is found between symptoms of hypersensitivity and levels of antigen-specific IgE (Aas and Johansson, 1971; Berg et al., 1971; Norman et al., 1973; Foucard and Johansson, 1976). Measurements of specific IgE generally agree with the results of skin and challenge tests, although variability still exists. The amount of non-specific or irrelevant IgE simultaneously present apparently plays a minor role, if any, in determining allergic sensitivity to a given antigen (Schellenberg and Adkinson, 1979).

Experimental elicitation of IgE. As with antibodies of other classes, elicitation of IgE in experimental animals requires antigens of large molecular weight, such as proteins and carbohydrates greater than 1000 daltons. Smaller molecules generally are not immunogenic unless they are conjugated with a carrier molecule of sufficient size.

Carriers selected for experimental conjugation with haptens include a variety of proteins, among them serum albumins, hemocyanins, ovalbumin, globulins, and thyroglobulins. For stimulation of IgE production, keyhole limpet hemocyanin (KLH) is a frequent choice.

The density of haptenic groups on the carrier can influence the antibody response. Too few or too many haptens per carrier molecule may produce no response or a brief response which can not be boosted (Klause and Cross, 1974; Quijada et al., 1974).

Erlanger (1980) noted that his group has never failed to obtain a response with as few as two haptens per carrier molecule, although a longer period of sensitization may be required for equivalent antibody titers.

Little and Eisen (1967) and Erlanger (1980) have reviewed the methods used to covalently bind haptens to the available carboxyl, amino, imidazo, phenolic, and/or sulfhydryl groups of proteins. The methods for conjugating haptens with carboxyl groups are described briefly here.

Haptens with carboxyl groups can be converted to acid anhydrides which react directly with amino groups. Carbodiimide coupling agents can also be used to form a peptide bond between carboxyl and amino groups. Water-soluble carbodiimides such as 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC) permit such coupling to proteins in aqueous solution. Other peptide coupling agents such as 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) are also effective (Fatori and Hunter, 1980). Reactions involved in conjugations with an acid anhydride derivative and with carbodiimide and EEDQ coupling agents are shown in Figure 2. Carboxyl groups can also be converted to the azide, the acid chloride, or the N-hydroxysuccinimide ester which react directly with protein.

To induce high levels of specific IgE, antigen is administered with an adjuvant. Jarrett (1978) and Pauwels et al. (1979) have found that aluminum hydroxide or Bordetella pertussis given with antigen to rats produces a primary IgE response which can be

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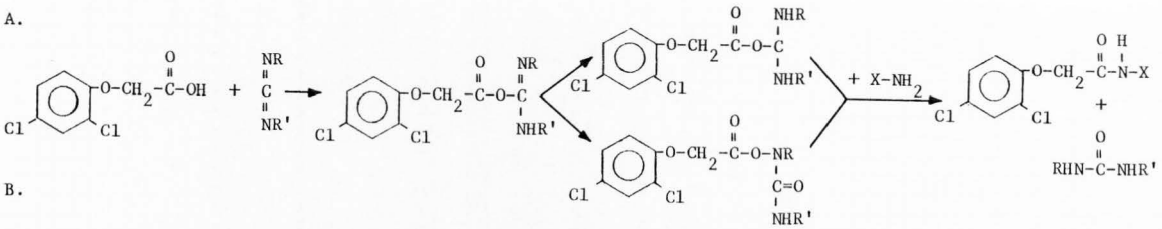

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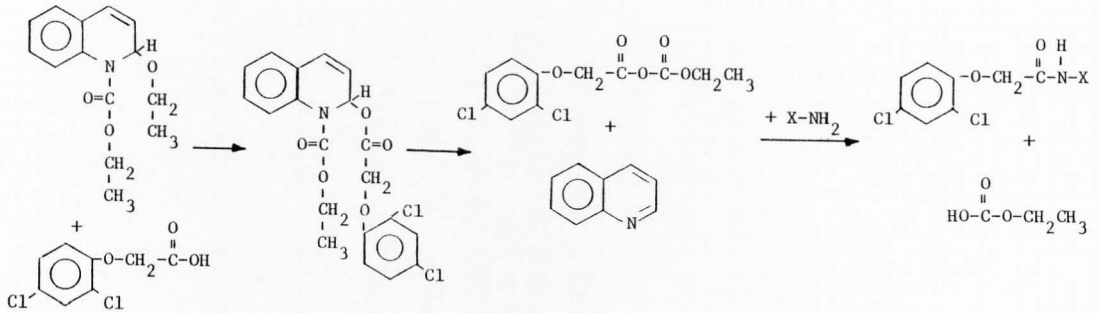

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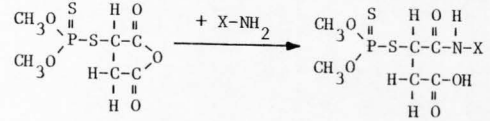


boosted with a subsequent antigen dose whereas using Freund's complete adjuvant, the primary response could not be boosted significantly. Infection with the nematode Nippostrongylus brasilienses (N.b.) stimulates total IgE and also IgE responses to unrelated antigens presented during infection (Jarrett, 1978). Similar results have been found in mice, with aluminum hydroxide and N.b. infection particularly effective as adjuvants (Revoltella and Ovary, 1969; Kojima and Ovary, 1975).

Measurement of IgE. The concentration of IgE in the serum of both normal and allergic individuals is so low that methods for quantifying IgE must be quite sensitive. Serum IgE specific for an antigen can be measured using the passive cutaneous anaphylaxis (PCA) test, the radioallergosorbent test (RAST), or the enzyme-linked immunosorbent assay (ELISA).

The PCA test was originally developed by Ovary (1958, 1964). In the PCA test, serum containing specific IgE against an antigen is injected intradermally into the shaved back of a previously unsensitized recipient animal. The IgE in the sample binds to membrane receptors on mast cells in the skin, thus passively sensitizing that region of the skin. After a suitable sensitization period, the recipient is given an intravenous injection of multivalent antigen and a dye such as Evans blue which binds to albumin and is normally restricted to the circulation. When the antigen reaches the IgE-bearing mast cells, it binds to the Fab portions of the IgE molecules. The crosslinking of the immunoglobulins by the antigen triggers the release of histamine, SRS-A,

and other vasoactive amines. The blood vessels in the immediate area dilate in response, allowing the area of anaphylaxis to be seen due to the outpouring of plasma containing the dye into the area. The PCA test using mouse sera originally called for mice as the recipient animal (Ovary, 1958, 1964). The use of rats as recipients was introduced by Mota and Wong (1969) and studied further by Bach and Braskler (1973), Ovary et al., (1975), and Okudaira et al. (1980). The rat PCA reaction is widely used for the measurement of mouse IgE antibody. Mota and Wong (1969) found that rat skin was sensitized by IgE but not mouse IgG_1 antibody which sensitizes homologous tissues. The specificity of the rat PCA test for mouse IgE was also indicated by studies showing that mouse IgE but not IgG_1 or IgG_2 bound to rat mast cells (Konig et al., 1974). The rat PCA requires a short sensitization period (a minimum of two hours), exhibits very clear blueing reactions, and easily permits 50 or more samples to be tested per rat.

The RAST procedure for measuring specific antibodies is shown in Figure 3. The allergen coupled to an insoluble matrix binds IgE antibody for the specific allergen present in the serum sample. Unbound antibody is removed by washing. Next, anti-IgE antibody labelled with ^{125}I is added and binds to the previously bound IgE. Following final washings, the radioactivity on the matrix is counted as a measure of the specific IgE in the serum sample. This test was originally described by Wide et al. (1967) who measured human serum IgE employing Sephadex beads as the matrix. Cellulose filter paper disks have been used as the matrix

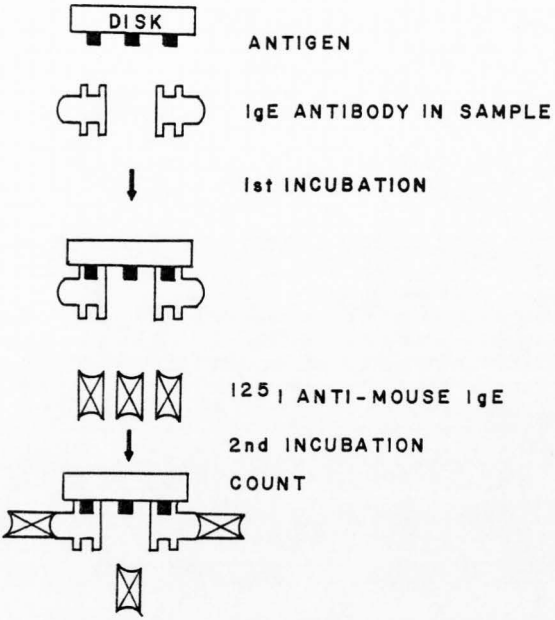


Figure 3. Diagram of the paper radioallergosorbent test (PRAST) for specific IgE (from Bennich et al., 1978).

in tests for specific IgE in rats (Pauwels et al., 1977a; Karlsson et al., 1979a) and in mice (Kelly et al., 1980). When paper disks are used, the assay is called the paper radioallergosorbent test (PRAST). The RAST and PRAST have been found to be specific for antibodies of the IgE class and at least as sensitive and accurate as the PCA assay. In addition to RAST assays for specific IgE, radioimmunoassays for total IgE have been developed that rely on competitive binding of labelled IgE and sample IgE (Bennich and Johansson, 1971; Gleich et al., 1971; Ishizaka et al., 1976) and noncompetitive binding of IgE in double antibody assays (Ceska and Lundkvist, 1972; Pauwels et al., 1977b; Karlsson et al., 1979b; Kelly et al., 1979).

ELISA procedures for antigen-specific IgE in human sera have been described by Weltman et al. (1976) and Vos et al. (1980). The wells of microtiter plates or cuvettes are coated with antigen and the sample added. Following incubation, anti-IgE antibody conjugated to an enzyme such as horseradish peroxidase is added to the wells. After an incubation period, a conventional substrate such as 5-aminosalicylic acid with hydrogen peroxide is added and the reaction product measured spectrophotometrically. A double antibody ELISA for total serum IgE has also been described by Hoshi et al. (1980). Assays that rely on enzymes linked to antibody have the advantage of sensitivity without the use of radioactive compounds and do not require the use of a large number of recipient animals necessary for the PCA assay. However, the

greatest sensitivity and accuracy requires a spectrophotometer modified to read microtiter plates.

Regulation of IgE antibody synthesis. Whether or not an animal's B lymphocytes produce specific IgE when exposed to an antigen appears to be controlled by T lymphocytes (Katz, 1978, 1980).

T lymphocytes must be present for the development of an IgE antibody response. This requirement was demonstrated by Okumura and Tada (1971a) who showed that rats thymectomized as neonates could not produce IgE antibody, and by Taniguchi and Tada (1974) who showed that thymectomized rats could produce IgE if injected with thymocytes from syngeneic rats.

T lymphocytes are also responsible for a suppressive mechanism which limits the production of IgE following sensitization. Treatment of a rat or mouse with whole body radiation (Tada et al., 1971; Chiorazzi et al., 1976), adult thymectomy or splenectomy (Okumura and Tada, 1971a), or administration of immunosuppressive drugs such as cyclophosphamide (Taniguchi and Tada, 1974; Chiorazzi et al., 1976) suppresses T lymphocytes and enables the animal to produce high quantities of IgE. Transfer of syngeneic thymocytes or spleen cells reconstitutes the suppressive mechanism and IgE antibody synthesis is depressed (Okumura and Tada, 1971b).

The mechanisms by which T lymphocytes regulate the production of IgE by B lymphocytes are not well defined. However, several soluble factors that enhance or suppress in vivo or in vitro IgE

synthesis have been described and are reviewed by Katz (1980). Factors include those isolated from spleen cells, serum, and ascites fluid as well as from cultures of lymphocytes, mesenteric lymph node cells, and spleen cells.

The level of IgE response differs among various inbred strains of mice (Levine and Vaz, 1970). The "low"-responder strains of mice such as C57BL/10, SJL, and SWR produce a weak or absent IgE response. "High"-responder strains, such as CBA, A/J, AKR and BALB/c produce relatively high levels of IgE. The genetic control of IgE responses is linked to the H-2 major histocompatability complex (MHC) in mice, with $H\text{-}2^{d}$ and $H\text{-}2^{k}$ alleles corresponding to high responders and other H-2 alleles to low responders. The low responder strains are believed to have an intact suppressive T cell subpopulation.

As mentioned above, infestation with certain helminth parasites such as N.b. induces synthesis of high levels of IgE against other antigens presented concurrently, as well as to the helminth. Katz (1978, 1980) has suggested that disturbance of the suppressive or "damping" mechanisms that normally limit IgE production such as by a parasite infestation, upper respiratory viral infection, or use of immunosuppressive drugs would result in excessive IgE production to another sensitizing antigen, thereby producing allergic symptoms. This concept has been named "allergic breakthrough" and has been investigated experimentally in rats by Katz.

Delayed-type hypersensitivity mediated by T lymphocytes

Description. Following exposure to an antigen, the delayed-type response in a sensitized individual reaches a maximum at 24 to 48 hours rather than in a matter of minutes to hours as described above for IgE-mediated hypersensitivity. The resulting inflammatory lesion is erythematous and infiltrated with lymphocytes and macrophages. Sensitivity to a particular antigen can be transferred from a sensitized to a previously unsensitized animal by T lymphocytes. Since transfer is accomplished by cells rather than serum, delayed-type hypersensitivity is grouped with cell-mediated immune reactions. Delayed hypersensitivity has been recently reviewed by Turk (1980) and Hay (1979).

Induction of contact sensitization is believed to require the hapten to penetrate the skin and react with proteins or cells to form a hapten-self carrier conjugate. Thus, lipophilic, highly reactive compounds such as dinitrofluorobenzene are excellent sensitizers. The exact nature of the carrier remains the subject of investigation: skin proteins, erythrocytes, serum proteins, lymphocytes, and macrophages have been considered. A macrophage-like cell found in the epidermis, the Langerhans cell, has been most recently proposed as the carrier (reviewed by Streilein et al., 1980). The Langerhans cell has the following characteristics: 1) it can process and present antigen in vitro, 2) it appears to derive from bone marrow rather than epithelium or neural crest, 3) it is the only epidermal cell to express cell surface determinants encoded by the immune response region of

the MHC, and 4) it is found beneath the dermis, along relevant lymphatic drainage routes, and in close opposition to lymphocytes during delayed skin reaction.

Following its formation, the hapten-carrier must encounter T-lymphocytes, either in the skin, the lymphatics, or the regional lymph nodes. The T-lymphocytes proliferate and differentiate into memory cells and effector cells (T_{DTH}).

Upon epicutaneous reexposure of a sensitized animal to the hapten, the elicitation (efferent) phase is initiated. Effector T cells interact with hapten in the skin and release lymphokines at the challenge site. The lymphokines include migration inhibitory factor which limits the outward migration of macrophages, chemotactic factor which attracts macrophages and lymphocytes, mitogenic factors which cause blast transformation of lymphocytes, macrophage activating factor, lymphotoxin which kills a variety of cells, and transfer factor which imparts antigen reactivity to unsensitized lymphocytes. T cells also interact with local mast cells which release 5-hydroxytryptamine, causing vascular changes which allow diapedesis of cells into the area (Gershon et al., 1975; Askenase et al., 1980). The challenged area becomes swollen and red, and contains an accumulation of lymphocytes and macrophages.

Measurement. Contact sensitivity in humans is diagnosed on the basis of distribution of the lesions and patient history and confirmed with patch testing. In patch tests, an acutely nonirritating concentration of the suspected contact sensitizer is

applied to the patient's skin and covered with an occlusive dressing. An eczematous reaction at 48 hours is considered positive.

In guinea pigs, sensitization by contact sensitizing agents may be produced by and later elicited by epicutaneous application (Buehler, 1965; Maguire, 1973), intradermal injection (Draize et al., 1944), or a combination of applications and injections (Magnusson and Kligman, 1970). The skin reactions are graded in terms of erythema, induration or edema, vesiculation, and necrosis.

Contact hypersensitivity in the mouse can be measured by several tests. Following challenge of the ear, swelling can be measured with an engineer's micrometer (Asherson and Ptak, 1968) or the ear can be weighed (Corsini et al., 1979). The mouse may be injected prior to challenge with a radiolabelled compound which is incorporated into the cells that subsequently migrate to the challenged ear (Vadas et al., 1975; Sabbadini et al., 1974). The magnitude of the hypersensitivity response can be determined based on differential counts of radioactivity in ears challenged with sensitizer versus those challenged with solvent. Histologic examination of the challenged ear provides additional information.

Regulation. The control mechanisms of contact sensitivity have been extensively studied, primarily in the mouse and guinea pig. Regulatory elements that prevent, enhance, or suppress hypersensitivity include suppressor T cells, soluble suppressor factors, "clone inhibition", anti-idiotypic antibodies, and the MHC

characteristics of the strain. These elements are diagrammed in Figure 4. Certain drugs such as cyclophosphamide also have been used to regulate the immune system. These control factors will be discussed briefly. More detailed information can be found in Hay (1979), Turk (1980), Claman and Miller (1980), Claman et al. (1980a, 1980b), and Polak (1980).

Animals can be made tolerant to a hapten by i.v. injection of free hapten, injection of in vitro hapten-modified syngeneic lymphoid cells, feeding of repeated doses of hapten, or applications of supraoptimal doses on the skin. Tolerance to the hapten can be transferred to a naive animal by the transfer of T cells. Transfer experiments using donors tolerant to a variety of haptens including DNFB, dinitrobenzenesulfonate, picryl chloride, and oxazolone have demonstrated the existence of at least two types of T suppressor cells (T_s). One type inhibits the elicitation or afferent phase and one inhibits the efferent phase of contact sensitivity. An additional T cell (T_{aux}) is believed to be required for the T_s efferent to be capable of acting on T_{DTH}. Genetic restriction on the induction and expression of T_s are located in the K and D ends of the MHC.

T_s cells may act in part through the release of macromolecules called soluble suppressor factors. These chemicals are released into culture media by lymphoid cells, are antigen-specific, and suppress contact sensitivity by suppressing the effector functions of T_{DTH}. The soluble suppressor factors carry determinants coded

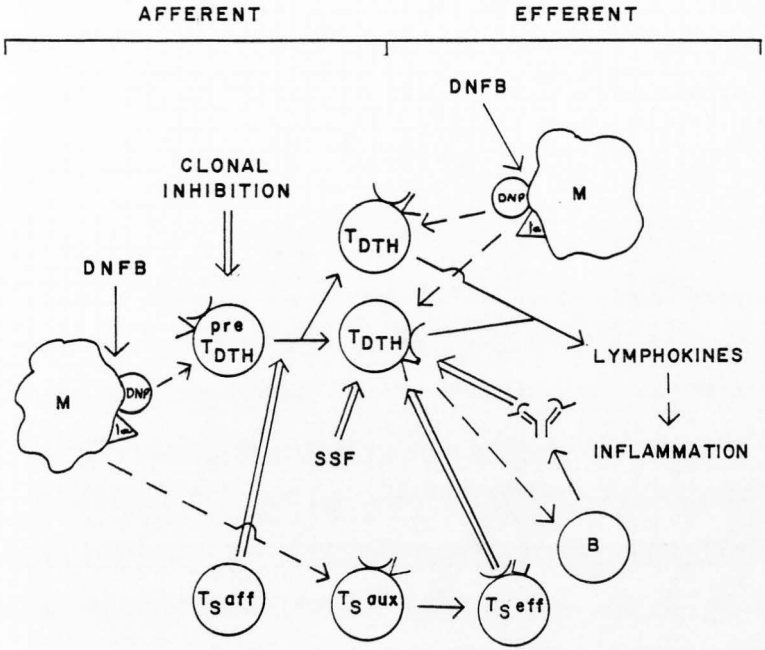


Figure 4. Schematic representation of the regulation of contact sensitivity in mice, adapted from Claman et al. (1980b). The M represents a macrophage; T_{DTH}, T lymphocytes that effect delayed-type hypersensitivity; $T_S$aff, afferent suppressor T lymphocytes; $T_S$eff, efferent suppressor T lymphocytes; $T_S$aux, auxiliary suppressor T lymphocytes; B, B lymphocytes, SSF, soluble suppressor factor; DNFB, dinitrofluorobenzene; DNP, dinitrophenyl group; Ia, membrane component determined by the immune response region of the major histocompatability complex.

by genes in the I-C subregion of the MCH and apparently are aimed at hapten and products of the MHC.

The rapid decline in contact sensitivity by about the twelfth day after sensitization in some strains of mice has been attributed to anti-idiotypic antibodies induced by antigenic determinants on specific T_{DTH}. The antibodies interact with the idiotype of the T receptor which results in suppression. This B cell-mediated suppression can be transferred with B cells or with serum containing antibodies with affinity for anti-hapten antibodies but not for hapten. This rapid decline in contact hypersensitivity is not seen in all strains or guinea pigs, which may retain their sensitivity for a longer period of time or even a lifetime.

Tolerance to a hapten can also exist which is not transferable with either serum or cells, and which resists other attempts to demonstrate T_S cells, including adult thymectomy, splenectomy, and pretreatment with cyclophosphamide. Claman et al. (1980a) hypothesized that the tolerance is due to inhibition of T cell clones required for expression of contact hypersensitivity. The mechanism of this "clonal inhibition" is not known, but could occur by direct interaction between a "tolerogen" and the T_{DTH}, by blockade of the receptors of the T_{DTH}, or by some form of clone deletion.

Chemicals such as cyclophosphamide that affect cell division have marked effects on contact sensitivity. The particular response depends on the dose and the timing of treatment. For

instance, cyclophosphamide pretreatment eliminates T_s efferent precursor cells or inducers and B cells, causing an increase in contact sensitivity. In contrast, cyclophosphamide administered at the time of sensitization causes tolerance due to elimination of T_{DTH} activated by the sensitization procedure.

Allergic Hypersensitivity to Pesticides

There are reports of hypersensitivity in humans exposed to a variety of pesticides (see the partial listing in Table 1). Most of these reports describe cases of allergic contact dermatitis and/or positive patch tests with occasional reports of respiratory symptoms in persons occupationally exposed to pesticides. Currently, evaluation of allergic hypersensitivity to pesticides in humans is based on patient history and skin tests. No evaluations of serum IgE using RAST or ELISA procedures in human hypersensitivity to pesticides have been reported, even though the presence of specific IgE has been shown to correlate with a number of human allergies.

The pesticides 2,4-D, malathion, and captafol have been reported to cause contact hypersensitivity and/or respiratory symptoms in humans. The limited information published on the effects of these chemicals on the immune system is reviewed in the following sections.

2,4-D

2,4-D (2,4-dichlorophenoxyacetic acid, Figure 5), in its formulations as esters, and sodium, amine, and lithium salts is

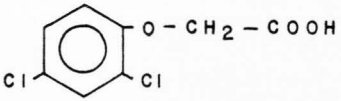
Table 1. Reports of human hypersensitivity to pesticides.

Pesticide	Reference
Actellic	Bainova et al., 1978
Benomyl	Fregert, 1967b; Savitt, 1972; Nomura et al., 1976; Hanada et al., 1977
Butyphos	Mirakhmedov and Yusupov, 1973
Captan	Fregert, 1967a; Horiuchi et al., 1977
Captafol	Takamatsu et al., 1968; Arimatsu, 1970; Verhagen, 1974; Camasara, 1975; Kambe et al., 1976; Groundwater, 1977; Hanada et al., 1977; Horiuchi et al., 1977; Peoples et al., 1978; Stoke, 1979; Horiuchi and Ando, 1980; Matsushita et al., 1980
Cartap	Kambe et al., 1976
CDAA	Spencer, 1966
Chlorfenvinphos	Kambe et al., 1976
Chlorothalonil	Horiuchi et al., 1976; Kambe et al, 1976; Hanada et al., 1977; Horiuchi and Ando, 1977; Horiuchi et al., 1977; Horiuchi et al., 1978; Horiuchi and Ando, 1980; Spindeldreier and Deichmann, 1980
Cyanophos	Kambe et al., 1976
Cyanphenphos	Kambe et al., 1976
2,4-D	Balo-Bango et al., 1973; Jung and Wolf, 1978; U.S. Environmental Protection Agency, 1980
DDT	Vanat and Vanat, 1971; Mirakhmedov and Karimov, 1972
Diazinon	Kambe et al., 1976
Dichlorvos	Fregert, 1967b; Kambe et al., 1976; Horiuchi et al., 1978; Horiuchi and Ando, 1980
Dicofol	Horiuchi et al., 1976
Dicotex	Telegina and Bikbulatova, 1970
Dimite	Horiuchi et al., 1976
Dinofen	Bainova et al., 1978
Fenitrothion	Kambe et al., 1976
Formothion	Mirakhmedov and Karimov, 1972; Kambe et al., 1976; Karimov, 1979
Fundazol	Bainova et al., 1978
Karathane	Mazzella di Bosco, 1970
Leptophos	Kambe et al., 1976
Lindane	Mirakhmedov and Karimov, 1972; Yamamoto et al., 1973

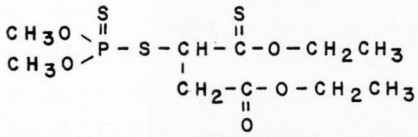
Table 1. Continued

Pesticide	Reference
Malathion	Milby and Epstein, 1964; Kligman, 1966
Mancozeb	Horiuchi et al., 1977; Kleibl and Rackova, 1980
Maneb	Laborie et al., 1964; Kambe et al., 1976; Nomura et al., 1976; Horiuchi et al., 1977; Horiuchi et al., 1978; Nater et al., 1979; Nater, 1979; Horiuchi and Ando, 1980
Methomyl	Kambe et al., 1976
Methyl mercaptophos	Mirakhmedov and Yusupov, 1973
Naled	Edmundson and Davies, 1967
Nematin	Jung and Wolff, 1970; Schubert, 1978
Nicotine sulfate	Horiuchi et al., 1977; Horiuchi and Ando, 1980
Nitrit	Fregert, 1967c
Nitrofen	Hanada et al., 1977
Omite	Nishioki et al., 1970
Ovex	Horiuchi et al., 1976
Paraquat	Horiuchi et al., 1976; Horiuchi et al., 1978; Horiuchi and Ando, 1977; Horiuchi et al., 1978
PCNB	Kambe et al., 1976
Phaltan	Fregert, 1967a
Phenmedipham	Nater and Grosfeld, 1979
Phenthoate	Kambe et al., 1976
Salithion	Horiuchi et al., 1977; Horiuchi et al., 1978
Schradan	Mirakhmedov and Yusupov, 1973
Thiometon	Mirakhmedov and Yusupov, 1973; Kambe et al., 1976
Thiophanate	Nomura et al., 1976; Hanada et al., 1977
Thiram	Baer and Rosenthal, 1954; Blank, 1956; Shelley, 1964; Kligman, 1966
Trifluralin	Horiuchi et al., 1976; Kambe et al., 1976; Horiuchi and Ando, 1977
Zineb	Laborie et al., 1964; Horiuchi et al., 1976; Kambe et al., 1976; Hanada et al., 1977; Zorin, 1970

A.



B.



C.

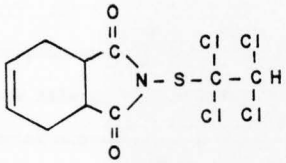


Figure 5. Chemical structures of A. 2,4-D, B. malathion, and C. captafol.

the most commonly used phenoxy herbicide, with, for example, 18.3 million kg used in the U.S. in 1974 by agriculture, industry, and government (Keil et al., 1977). 2,4-D formulations are registered for use on a variety of crops and in non-crop areas for postemergent control of broadleaf weeds. Human health hazards associated with the use of 2,4-D have been relatively insignificant (Bovey and Young, 1980). However, 2,4-D has been implicated in occasional reports of hypersensitivity reactions in humans.

Balo-Bango et al. (1973) described a case involving severe bullous dermatitis in a man following exposure to a cornfield sprayed with 2,4-D. In another report, a female orchard worker in contact with the herbicide Selest 100, a mixture of esters of 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in diesel oil, demonstrated a severe allergic eczema confirmed by patch tests whereas the oil carrier alone gave no response (Jung and Wolf, 1978). Tests to separate responses to 2,4-D, 2,4,5-T, or possible contaminants were not conducted. A search of the pesticide incidents involving 2,4-D reported to the Pesticide Incident Monitoring System (PIMS) of the U.S. Environmental Protection Agency from 1966 to 1980 yielded 13 cases of skin rash or dermatitis, one case of allergic nasopharyngitis, and one case of mild anaphylactic response associated with the use of 2,4-D alone (U.S. Environmental Protection Agency, 1980). In a generalized report of incidents due to agricultural chemicals in Japan, Wakatsuki (1978) stated that dermatitis to chlorophenoxy acids is often a problem. Although confirmation of the involvement of 2,4-

D is not certain in these cases, these reports provide circumstantial evidence for the ability of 2,4-D to produce allergic responses in humans.

Studies of the effects of 2,4-D on the immune system of experimental animals apparently have not been reported.

Malathion

Malathion (diethyl mercaptosuccinate, S-ester with 0,0-dimethyl phosphorodithioate, Figure 5) is an organophosphate used to control a wide variety of insects, including aphids, spider mites, house flies, and mosquitoes. It has a relatively low acute toxicity for mammals due to the ability of mammals to detoxify the molecule (Krueger and O'Brien, 1959). Use of malathion in the U.S. in 1974 was estimated at 4.3 million kg (Keil et al., 1977).

Malathion has been shown to sensitize human volunteers (Milby and Epstein, 1964; Kligman, 1966). Malathion at a concentration of 10% in alcohol or water or a concentration of 0.5 to 1.5 % in diesel oil acted as a strong sensitizer, inducing a positive patch test in 13 of 29 and eight of 23 volunteers, respectively, when challenged with 1% malathion in alcohol (Milby and Epstein, 1964). Five subjects who were very sensitive to malathion had strong reactions to a dilution of one part per million in acetone. The sensitizing potential of 1% and 0.1% solutions in alcohol was considerably weaker than that of the 10% solution. Only one of 15 persons exposed to a 0.1% solution became sensitized.

A field survey of two groups occupationally exposed to malathion discovered four of 157 mosquito abatement district workers

and two of 43 poultry ranchers who reacted to patch tests using 1% malathion (Milby and Epstein, 1964). Five of the six people who reacted had previous histories of undiagnosed dermatitis.

Kligman (1966) attributed the failure of malathion to sensitize more exposed workers to the low concentration normally applied and to transient skin contact under regular use patterns.

Magnusson and Kligman (1970) compared the sensitizing potential of malathion in the guinea pig maximization test employing both intradermal injections and epidermal applications for sensitization with the sensitizing potential of malathion administered by both modes separately. Intradermal injections alone or combined with topical exposures using a 10% solution produced sensitization in over half of the animals tested. Topical applications of a 10% solution produced sensitivity in four of 24 guinea pigs while zero of 24 animals reacted to a 25% solution. These researchers also tested malathion using the procedure described by Draize et al. (1944) and found that no sensitization was produced when a 0.1% solution was injected intradermally.

Vijay et al. (1978) investigated the ability of a conjugate of the anhydride of a product of carboxyesterase action, mercaptosuccinate S-ester with O,O-dimethyl phosphorodithioate (also named malathion metabolite anhydride, MMA), conjugated with bovine serum albumin (BSA) to elicit IgE antibodies. They used single intraperitoneal injections of 1.0 and 2.0 mg of MMA-BSA to sensitize Wistar Furth rats. The highest mean PCA titer obtained was 32, 18 days after immunization in inbred rats that were dosed with 1.0 mg

of conjugate. The investigators did not attempt to elicit a secondary response.

In further studies on the effects of malathion on the immune system, Vijay et al. (1979) dosed Wistar Furth rats orally with 2 or 10 mg of malathion daily for five days. The malathion treatment substantially reduced the IgE antibody produced following immunization with an extract of a T1 colony of Neisseria gonorrhoeae strain GC6. Slight suppression of the production of anti-MMA IgE antibodies was observed. Antibodies against sheep red blood cells were also slightly reduced in the rats dosed at the higher level. The researchers suggested that malathion impairs thymus-dependent immunity and acts as an immunosuppressant. Suppression of the humoral response to Salmonella typhi was also reported by Desi et al. (1978). Rabbits dosed orally with 5, 10, 25, 50, or 100 mg per kg, five times weekly for six weeks, showed a dose-related decrease in antibody titers as determined by tube agglutination.

The MMA-BSA conjugate prepared using the procedure described above injected with Freund's complete adjuvant was capable of eliciting antibodies with high hemagglutination titers (Centeno et al., 1970). Another malathion-protein conjugate prepared by reacting malathion acyl chloride with fibrinogen also elicited measureable hemagglutination titers (Haas and Guardia, 1968).

Captafol

The fungicide captafol (difolatan; N-[tetrachloroethylthio]-tetrahydrophthalimide; Figure 5) is applied to fruits and vegetables and also to timber to control a variety of mildews, blights, molds, and other fungi. Use of captafol in the U.S. in 1974 was estimated at 0.9 million kg (Keil et al., 1977).

Captafol has been reported to cause both irritant and allergic contact dermatitis. Farm workers in California (Peoples et al., 1978) and in Japan (Takamatsu et al., 1968; Kambe et al., 1976; Hanada et al., 1977; Horiuchi et al., 1977; Horiuchi and Ando, 1980; and Matsushita et al., 1980), workers in timber treatment plants (Stoke, 1979), and coffee pickers in Kenya (Verhagen, 1974) have suffered from dermatitis caused by captafol, sometimes accompanied by conjunctivitis or bronchitis. In these reports, cases of irritant contact dermatitis were not distinguished from allergic contact dermatitis. Camasara (1975) performed patch tests using a 1% concentration of captafol on seven Spanish chemical packers who had developed urticarial reactions and marked asthmatic dyspnea and found positive reactions in four workers. An American welder who worked in plants that distributed captafol also developed vesiculation and edema of the face and hands accompanied by wheezing. Patch tests were positive for captafol (Groundwater et al., 1977).

Studies by Kasai and Sugimoto (1973) with guinea pigs showed that 80% WP, technical, and purified captafol caused dermatitis, with the impure products giving stronger reactions.

STATEMENT OF THE DISSERTATION PROBLEM

Research Objectives

These studies were undertaken to investigate allergic hypersensitivity in mice to three widely used pesticides, the herbicide 2,4-D, the insecticide malathion, and the fungicide captafol, which have been reported to cause allergic reactions in exposed humans. Three main objectives governed the approaches taken to the problem.

The primary objective of the research was to elicit and characterize hypersensitivity to these pesticides. Since hypersensitivity may be mediated by specific IgE antibodies in immediate hypersensitivity or by activated T lymphocytes in delayed-type hypersensitivity, it was necessary to conduct studies to evaluate both types. The elicitation of specific IgE required the synthesis and administration of pesticide-protein conjugates and was followed by the analysis of blood samples using the PCA and PRAST assays. Several methods of evaluating delayed-type hypersensitivity were used in mice exposed epicutaneously. These methods consisted of evaluating the increase in ear thickness to quantitate swelling, the accumulation of radiolabelled cells to measure the cellular component of the response, and histological preparations of challenged ears. The original intent was to investigate 2,4-D and malathion by these procedures. Captafol was later included in the studies of contact hypersensitivity.

To permit tests for specific IgE in the sera of mice to be potentially useful for testing sera of exposed humans, it was necessary to develop a second assay in addition to the PCA test. The PRAST procedure was selected due to its sensitivity and previous success in clinical determinations of human allergies to a variety of substances. Thus, the second objective was to develop the PRAST for determination of IgE specific to 2,4-D and malathion.

Thirdly, these studies were concerned with the use of the BALB/c mouse as an animal model for studying hypersensitivity. Guinea pigs have frequently been selected as the animal in which to study hypersensitivity, particularly by toxicologists interested in assessing the contact sensitizing potential of new chemicals. On the other hand, a great deal of research on the control mechanisms regulating hypersensitivity has exploited inbred strains of mice. In addition, mice are cheaper, easier to house and maintain, and for these practical reasons may be preferred over guinea pigs for studying hypersensitivity if the ability to generate a hypersensitive response to a chemical can be demonstrated. The BALB/c strain of mice was evaluated by observation of its ability to mount an IgE response to haptens conjugated to a protein and by comparison of the results of the delayed-type hypersensitivity tests with the results of similar tests in guinea pigs reported in the literature.

MATERIALS AND METHODS

Animals and Materials

Animals

BALB/c female mice, 9 weeks old, and Sprague-Dawley rats, 300 to 650 g, were obtained from Simonsen Laboratories, Inc., Gilroy, CA. The animals were fed Wayne Lab Blox, Allied Mills, Chicago, IL and given access to water ad libitum.

Reagents and biochemicals

Pesticides and sensitizers. 2,4-dichlorophenoxyacetic acid (2,4-D) was obtained from the Aldrich Chemical Co., Milwaukee, WI and recrystallized from benzene (melting point 139 to 141 °C).

A secondary standard of malathion (diethyl mercaptosuccinate, S-ester with O,O-dimethyl phosphorodithioate, lot 3412-6) was a gift of the American Cyanamid Co., Princeton, NJ.

Captafol (N-[tetrachloroethylthio]tetrahydrophthalimide), analytical reference standard (lot no. 9226), was supplied by the Quality Assurance Section of the U.S. Environmental Protection Agency, Research Triangle Park, NC and had a purity of 99.6% as tested at the source.

Dinitrofluorobenzene (DNFB), 98% pure, was obtained from the Aldrich Chemical Co., Milwaukee, WI.

2,4-dinitrobenzene sulfonic acid from the Baker Chemical Co., Phillipsburg, NJ was recrystallized from 95% ethyl alcohol (melting point above 300 °C).

Chemicals used in the synthesis of MMA. Benzene (pesticide grade, water 0.05% maximum) and phosphorus pentasulfide from the Baker Chemical Co., Phillipsburg, NJ and methanol (pesticide grade, water 0.01% maximum) from the Fisher Scientific Co., Pittsburg, PA were used to synthesize O,O-dimethyl phosphorodithioate. Maleic anhydride from the Aldrich Chemical Co., Milwaukee, WI was then used to make the anhydride of mercaptosuccinate, S-ester with O,O-dimethyl phosphorodithioate. also called malathion metabolite anhydride (MMA).

Proteins. Bovine serum albumin (BSA), 15.4% nitrogen, was supplied by the Sigma Chemical Co., St. Louis, MO and was used for hapten-protein conjugates and the assay buffer in the paper radioallergosorbent assay. Keyhole limpet hemocyanin (KLH) was obtained from Pacific Bio-Marine Supply Co., Venice, CA and purified according to Garvey et al. (1977). The final purified KLH was dissolved in phosphate buffered saline (PBS, 0.15 M phosphate, pH 7.2) and sterilized by filtration. All saline solutions were 0.15 M NaCl. The protein concentration of the KLH solutions was calculated from Kjeldahl nitrogen determinations performed in duplicate based on a nitrogen content of 15.4% (Fairless et al., 1967).

Chemicals used in the determination of nitrogen. Nitrogen content of proteins and conjugates was determined using the micro-

Kjeldahl method (Horwitz, 1975). The following chemicals were reagent grade: sulfuric acid, hydrochloric acid, mercuric chloride, potassium sulfate, sodium hydroxide, sodium thiosulfate, boric acid, methyl red, and methylene blue.

Chemicals used in the determination of phosphorus. Total phosphorus content of MMA-protein conjugates was determined by the method of Fiske and Subbarow (1925). The chromogenic reagent, 1-amino-4-naphthol sulfonic acid from Eastman Organic Chemicals, Rochester, NY, was recrystallized prior to use. Sulfuric acid, ammonium molybdate, sodium sulfite, sodium bisulfite, monobasic potassium phosphate, and 30% hydrogen peroxide were reagent grade.

Radioactive materials. 5-[^{125}I]-iodo-2'-deoxyuridine ([^{125}I]-UdR), activity 5.3 Ci per mg, used in tests for delayed-type hypersensitivity was purchased from Amersham, Arlington Heights, IL. Radioiodination of anti-IgE was performed using ^{125}I, activity 17 Ci per mg, from New England Nuclear, Boston, MA.

Antibodies and materials used in PRAST. Purified goat anti-mouse IgE antibodies were purchased from Dr. K. A. Kelly, University of Manitoba, Canada. The antibodies were elicited using purified anti-ovalbumin monoclonal mouse IgE and isolated by affinity chromatography with purified anti-DNP monoclonal mouse IgE as the immunosorbent, according to Kelly et al. (1980). Chloramine-T and sodium metabisulfite used in the radioiodination of anti-IgE antibodies were reagent grade. Separation of labelled anti-IgE antibodies from the reaction mixture was performed on a

column of Bio-Gel P-60 from Bio-Rad Laboratories, Richmond, CA. Discs were punched from Whatman 541 hardened, ashless filter paper and were activated with cyanogen bromide. Sodium hydroxide, ethanolamine, and guanidine hydrochloride were reagent grade. The assay buffer consisted of reagent grade sodium azide, disodium phosphate, and dibasic sodium phosphate and Tween 20 and BSA from the Sigma Chemical Co., St. Louis, MO.

Other reagents. The coupling agents 2-ethoxy-1-ethoxy-carbonyl-1,2-dihydroquinoline (EEDQ) and 1-(3-dimethylamino-propyl)-3-ethyl-carbodiimide hydrochloride (EDC) were obtained from the Aldrich Chemical Co., Milwaukee, WI. Aluminum sulfate and sodium hydroxide used to prepare the adjuvant aluminum hydroxide were also reagent grade.

Methods

Preparation of conjugates

2,4-D-KLH. The coupling agent EEDQ was used to couple 2,4-D and KLH using a modification of the method of Fatori and Hunter (1980). The reaction was performed in an ice bath and the pH was monitored continually. 2,4-D (0.48 g) was dissolved in 2 ml of acetone:water (2:1) at pH 4.0. EEDQ (0.118 g) in 0.7 ml acetone was added to the 2,4-D and allowed to react for 10 min. The pH was maintained at 4.0 with 2 N HCl. The 2,4-D and EEDQ mixture was added drop-by-drop to a stirred solution of KLH (0.1675 g in 1.5 ml of PBS (0.15 M phosphate, pH 7.2) over 3 min. The pH was raised to 8.0 with 0.5 N NaOH and maintained for 2 hours. The

conjugate was extensively dialyzed against PBS (0.01 M phosphate, pH 7.2). A second conjugation was prepared and the two batches combined, sterilized by filtration, and analyzed.

Protein concentration was estimated by Kjeldahl nitrogen analyses. To determine the concentration of 2,4-D, the conjugate was first hydrolyzed in 7 N HCl in a boiling water bath for 10 hours. The sample was extracted twice with benzene, taken to dryness, and redissolved in methanol. The hydrolyzed conjugate was analyzed for 2,4-D using a Varian Model 5000 high-performance liquid chromatograph with a reversed-phase C-18 column using 35% acetonitrile and 65% water (pH 3.0) at a flow rate of 2.0 ml per min. Absorbance at 285 nm was recorded. All analyses were performed in duplicate.

2,4-D-BSA. The conjugation of 2,4-D and BSA was performed using the coupling agent EDC. The reaction was performed at room temperature. 2,4-D (0.2210 g) was dissolved in 2 ml dioxane and brought to pH 4.75 with 0.5 M NaOH. EDC (0.1917 g) was dissolved in 2 ml water, the 2,4-D solution added rapidly, and the pH maintained at 4.75 with 0.5 M NaOH. This mixture was added slowly to a solution of BSA (0.1675 g in 10 ml water) at pH 6.0 with rapid stirring. The time from addition of the water to the EDC to complete addition of the 2,4-D and EDC combination to the protein solution was found to be critical and was kept at approximately 5 min. Too rapid addition caused precipitation of the protein while too slow addition yielded a low degree of conjugation.

The pH of the mixture was brought to 6.0 and the volume to 20 ml with water. The mixture was allowed to react at room temperature for 1 hr and then at 4°C for 5 hr. The solution was then extensively dialyzed against PBS (0.01 M phosphate, pH 7.2). Seven batches of conjugate were made following this procedure, dialyzed, and combined. The conjugate was sterilized by filtration and analyzed in duplicate as described above for 2,4-D-KLH.

MMA-KLH and MMA-BSA. 0,0-dimethyl phosphorodithioate was prepared using the method of March et al. (1956). Phosphorus pentasulfide (35.2 g) was slurried into 40 ml of anhydrous benzene. The temperature of the mixture was maintained at 4°C and 28.8 ml of anydrous methanol was added dropwise. Following 3 hours of stirring, the solvent was removed under water aspirator pressure in a Roto-vap. The product was distilled at 42 to 44°C at 0.5 mm. The yield was 32.7 g.

The 0,0-dimethyl phosphorodithioate was used to synthesize MMA by the method of Centeno et al. (1970). In this procedure, 17.2 g of maleic anhydride was mixed with 27.9 g of 0,0-dimethyl phosphorodithioate and heated at 85°C for 16 hr. The red oil was used without further purification.

The conjugate of MMA with KLH was prepared using the method of Vijay et al. (1978). The anhydride of MMA (0.113 g) was dissolved in 2 ml of absolute ethanol and then added to 0.090 g of KLH dissolved in 15 ml of PBS (0.01 M phosphate, pH 7.4). The pH was maintained at 7.2 to 7.8 with 0.5 M NaOH. The mixture was stirred an additional 15 min at room temperature, held at 4°C

overnight, and then dialyzed extensively at 4°C against 0.05 N NaOH and then distilled water.

Five batches of MMA-BSA were prepared as described above, each using 0.600 g of the anhydride of MMA dissolved in 3 ml ethanol added to 0.480 g of BSA dissolved in 20 ml of PBS (0.01 M phosphate, pH 7.2). Following dialysis, the five batches were combined, placed in fresh dialysis bags, and concentrated by pervaporation.

The MMA-KLH and MMA-BSA were sterilized by filtration and then analyzed. The degree of conjugation was calculated from the concentration of phosphorus determined by the Fiske-Subbarow procedure and from an estimation of the protein concentration by Kjeldahl nitrogen analysis. Analyses were performed in duplicate.

Dinitrophenyl-KLH (DNP-KLH) and DNP-BSA. DNP-KLH and DNP-BSA were prepared as described by Little and Eisen (1967). Equal weights of potassium carbonate, protein, and 2,4-dinitrobenzene sulfonic acid were dissolved sequentially in suffucient water to bring the concentration of each to 20 mg/ml. The mixtures were stirred at room temperature for six hours. The solutions were dialyzed extensively against PBS (0.01 M phosphate, pH 7.2) and sterilized by filtration. The degree of conjugation was calculated from the estimated concentration of DNP groups based on absorbance at 360 nm and from an estimation of the protein concentration by Kjeldahl nitrogen analysis. Analyses were performed in duplicate.

Preparation of aluminum hydroxide

Aluminum hydroxide was prepared according to a slight modification of the method of Levine and Vaz (1970). Two N $Al_2(SO_4)_3$ was mixed with an equal volume of 2 N NaOH. The resulting gel was washed 10 times with distilled water and then homogenized for 3 min in a Waring blender. Solid NaCl was added to the gel to make a final concentration of 0.15 M NaCl and the pH was adjusted to 7.5 with 2 N NaOH. One ml samples of well-stirred gel were dried at 110 °C. The concentration was adjusted to 60 mg $Al(OH)_3$ per ml with 0.15 M NaCl.

Immunization with conjugates

2,4-D-KLH. Female BALB/c mice, 10 per group, were immunized with two intraperitoneal injections on Days 1 and 28 with 1, 10, or 100 ug of 2,4-D-KLH or 1 ug of DNP-KLH with 1 mg of aluminum hydroxide gel in 0.5 ml of sterile saline. The negative control group received aluminum hydroxide and saline alone. On Days 7, 14, 21, 36, and 43, 0.2 ml of blood was taken from the tail vein and mixed with 0.7 ml of sterile saline. The sample was allowed to clot at 4 °C overnight, then centrifuged, and the supernatant was taken to be a 1:8 dilution.

MMA-KLH. Immunization with 1, 10, or 100 ug of MMA-KLH or 1 ug of DNP-KLH was performed as described above on Days 1, 28, and 58. Blood samples were taken on Days 7, 14, 21, 36, 43, and 65 and handled as previously described.

Passive cutaneous anaphylaxis (PCA) reaction

The mouse sera were tested for 2,4-D-specific, MMA-specific, or DNP-specific IgE antibodies in Sprague-Dawley rats (Mota and Wong, 1969; Okudaira et al., 1980). One-tenth ml of serial two-fold dilutions of mouse sera were injected intracutaneously into the shaved dorsal skin of the rat. The reaction was induced after a 24 hr sensitization period by an i.v. injection of 4 mg of 2,4-D-BSA, MMA-BSA, or DNP-BSA in 1 ml of 1% Evans blue in saline. Thirty min after the challenge, the rats were sacrificed and the inside of the skin examined. A blueing reaction of 5 mm or greater was considered positive. Each serum dilution was tested in two rats. The PCA titer was expressed as the geometric mean plus or minus the geometric standard error of the reciprocal of the final dilution giving a positive blueing reaction in at least one rat.

PRAST

Preparation of paper disks. 2,4-D-BSA and MMA-BSA were coupled to paper disks for the solid phase of the PRAST using the method of Kelly et al. (1980). The conjugates were first dialyzed against 0.05 M $NaHCO_3$-Na_2CO_3, pH 10. Disks 5 mm in diameter were punched from Whatman 541 hardened, ashless filter paper and activated with cyanogen bromide (CNBr) according to techniques reported by Ceska and Lundkvist (1972). Five g of paper disks (approximately 2500 disks) were swelled in 50 ml of distilled water, the water decanted, and the disks added to 5 g of CNBr

freshly dissolved in 150 ml of distilled water. The pH was raised to 10.5 with 1 M NaOH and kept there until 25 ml were consumed. The liquid was drained off and the disks washed 12 times in 200 ml of 0.05 M $NaHCO_3$-Na_2CO_3 at pH 10 for 2 min each time. The discs were added to a solution of 250 mg protein conjugate in 100 ml buffer and stirred gently for 16 hr at 4°C. Following a wash with 200 ml of buffer for 10 min, the disks were slowly stirred for 3 hr with 200 ml of 0.5 M ethanolamine (pH 10) and then with 200 ml of 4 M guanidine hydrochloride (pH 4). Finally the disks were washed several times and equilibrated with assay buffer which consisted of 0.1% BSA, 0.5% Tween-20, 0.1% sodium azide, and 0.05 M disodium EDTA in PBS (0.01 M phosphate), pH 7.4. The disks were stored at 4°C in a small volume of assay buffer.

Radioiodination of anti-IgE antibodies. Anti-IgE antibodies were radioiodinated with ^{125}I using chloramine-T according to the method of Greenwood et al. (1963) as described by Kelly et al. (1979). Twenty-five ul of 0.5 M phosphate buffer, pH 7.5, 1.0 mCi ^{125}I (10 ul), and 25 ul (100 ug) chloramine-T were added sequentially to 25 ul (100 ug) anti-IgE antibodies. The solution was mixed for 45 seconds at room temperature. Then, 100 ul (240 ug) of sodium metabisulfite was added followed by 200 ul (2 mg) of carrier potassium iodide.. All reagents were prepared in 0.05 M phosphate buffer, pH 7.5. The sample and an additional 200 ul of carrier potassium iodide used to rinse the reaction vial were placed on a Bio-Gel P-60 column (1.6 x 30 cm) equilibrated with assay buffer to separate the ^{125}I-labelled anti-IgE from the

reaction mixture. Fractions (1 ml) of eluate were collected and counted on a Beckman gamma counter. Because the assay buffer contained 0.1% BSA, it was not possible to measure the amount of anti-IgE in the eluate. Therefore, it was not possible to determine the specific activity of the labelled anti-IgE antibodies.

Analysis of serum samples using PRAST. Each serum sample was diluted as required with assay buffer containing pooled normal rat serum (50:1). The sample (0.1 ml) was placed on an antigen-coupled paper disk previously washed once with one ml of assay buffer in a clear polystyrene Falcon culture tube. The disks were incubated at room temperature with gentle shaking for varying periods of time and then washed three times each with one ml of assay buffer. ^{125}I-labelled anti-IgE antibodies (0.2 ml) in assay buffer containing normal goat serum (10:1) were added to the tube and reacted for varying time periods at room temperature with gentle shaking. The disks were counted on a Beckman gamma counter for five min after being washed with one ml of assay buffer. Following experiments in which the times of incubation were varied from 1 to 24 hours, incubation times of 18 hours for reaction of the sample with the disk and of 24 hours for reaction of the labelled antibody with the disk were selected for use with the remaining samples. All samples were analyzed in duplicate. Results were reported as counts per minute (cpm).

Reaction conditions for the PRAST were optimized using pooled sera obtained at day 65 from mice immunized with 100 ug of MMA-KLH and at day 43 from mice immunized with 1 ug of 2,4-D-KLH. Other

serum samples analyzed were from mice that received various immunization schedules.

Epicutaneous sensitization and evaluation of delayed-type hypersensitivity (DTH)

Mice were pretreated two days prior to first sensitization with an intraperitoneal injection of cyclophosphamide, 200 mg per kg of body weight, in sterile saline (20 mg per ml) to augment the response or the equivalent volume of saline alone.

Groups of eight mice were sensitized by two daily applications of 25 ul of solution to the shaved abdomen. The chemical was spread with the side of the automatic micropipette tip within an area of approximately 2 cm^2. The following doses were administered in acetone:ethanol (4:1): 2,4-D - 11.8 and 118.4 mg/ml, malathion - 17.8 and 177.6 mg/ml, captafol - 18.7 and 37.4 mg/ml, DNFB - 5 mg/ml. The doses of 2,4-D and malathion are equivalent to 2 and 20 times and the doses of captafol are 2 and 4 times the dose of DNFB on a molar basis. Control mice were treated similarly with the solvent alone.

Six days after the second sensitization (day 8), the mice were challenged on the right ear with 20 ul of solvent and on the left ear with 20 ul of 2,4-D (11.8 mg/ml), malathion (17.8 mg/ml), captafol (18.7 mg/ml), or DNFB (2 mg/ml).

Following challenge, DTH was evaluated by measurements of ear thickness, ear histology, and the radioisotopic method of Vadas et al. (1975). Ten hours after challenge, each mouse received 2 uCi of [^{125}I]-UdR in 0.1 ml of sterile saline intravenously. Eighteen

hours later, ear thickness was measured with a Mitutoyo engineer's micrometer in units of 10^{-2} mm. The pinnae were cut off at the hair line, placed in buffered formalin in individual tubes, and counted on a Beckman gamma counter. The ratios of the thicknesses of the left ear to the right ear and of the counts per 5 min of the left ear to the right ear were calculated. For histology, the ears were cut and stained with hematoxylin and eosin and also toluidine blue for mast cells and basophils.

Other groups of ten mice received sensitizing applications of the test materials or solvent on the abdomen of days 1, 2, 8, 15, 22, and 29. On days 8, 15, 22, and 29, a blood sample was taken from each mouse as described previously. The sera were tested for 2,4-D-, MMA-, or DNP-specific IgE antibodies using the PCA test. Ten 2,4-D, malathion, captafol, or DNFB-treated mice and six solvent-treated mice were challenged on the ears six days after the final sensitization (day 35). Ear thickness was measured 24 hrs after challenge.

Statistical tests

The means of the test and appropriate control groups were compared using the t test for equal or unequal variances, as determined by F ratios (Brownlee, 1960).

The relation between the results of the PRAST and PCA test was measured by calculating the coefficient of determination (R^2) (Snedecor and Cochran, 1967).

RESULTS

Evaluation of Allergic Hypersensitivity to 2,4-D

Preparation of 2,4-D- and DNP-protein conjugates

The degrees of conjugation of the 2,4-D and DNP haptens with KLH and BSA are presented in Table 2. The KLH preparations did not yield as high a degree of conjugation as the BSA preparations, but the conjugates were considered adequate for sensitization.

Measurement of 2,4-D- and DNP-specific IgE antibodies using the PCA test

2,4-D-KLH injected intraperitoneally elicited IgE antibodies specific for 2,4-D following the second immunization as demonstrated by positive PCA reactions using 2,4-D-BSA as the challenge antigen (Figures 6 and 7). Within the range of dose levels tested, the highest titers were produced seven days after secondary immunization in the mice that were sensitized with 1 ug of 2,4-D-KLH. All the mice in that group produced detectable 2,4-D-specific IgE. Fewer mice responded to the 10 ug and 100 ug doses of 2,4-D-KLH. The geometric mean of the titers for the group receiving 10 ug was lower than that for the 1 ug group. The single responding animal that received 100 ug had titers that were within the range of the responses of the group that received 1 ug.

Table 2. Degree of conjugation for hapten-protein conjugates.

Conjugate	Degree of Conjugation[a]
2,4-D-KLH	5.0
2,4-D-BSA	13.3
MMA-KLH	9.0
MMA-BSA	12.8
DNP-KLH	10.6
DNP-BSA	27.5

[a] The number of 2,4-D, malathion metabolite anhydride (MMA), or dinitrophenyl (DNP) groups per 100,000 daltons of keyhole limpet hemocyanin (KLH) and per molecule of bovine serum albumin (BSA).

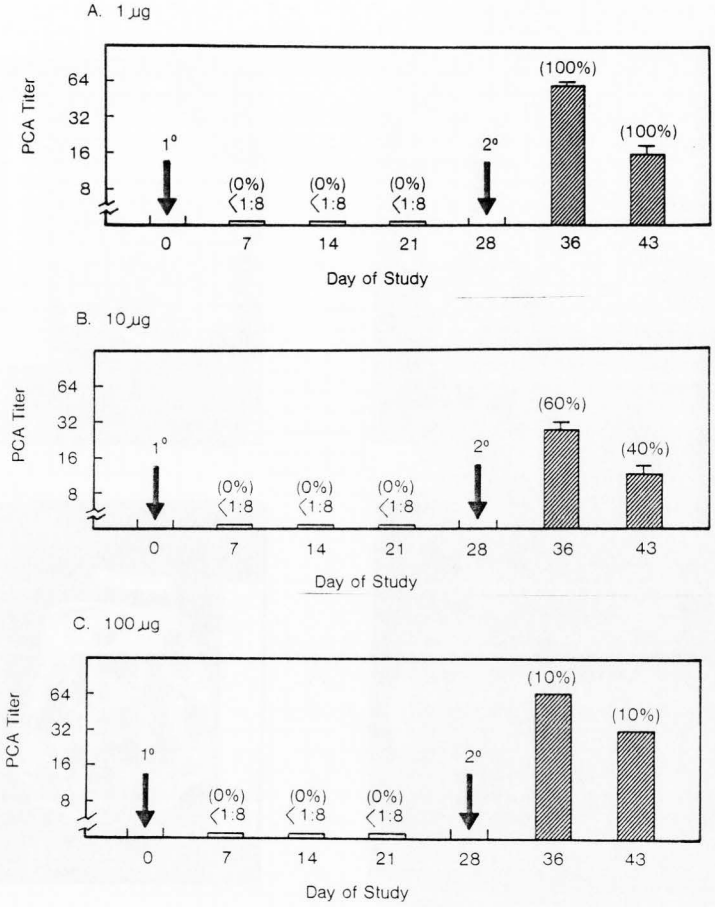


Figure 6. IgE antibody response specific for 2,4-D in female BALB/c mice. Primary (1°) and secondary (2°) doses of 2,4-D-KLH adminstered i.p. were A. 1 ug, B. 10 ug, and C. 100 ug. The bars represent the geometric mean $\pm$ the standard error of the PCA titer for mice producing detectable circulating IgE antibodies. The percentage of mice that produced detectable antibodies is shown at the top of each bar.

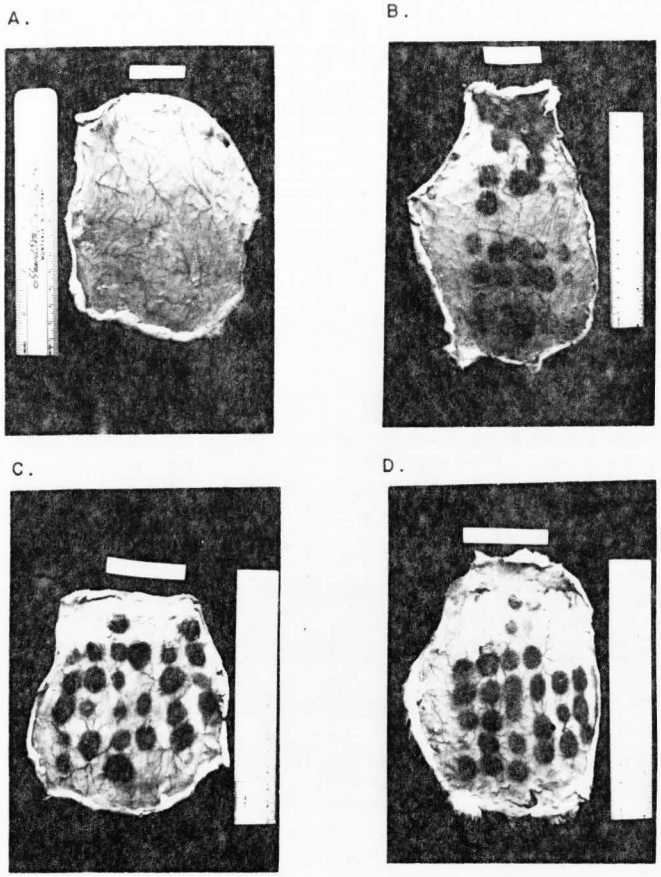


Figure 7. Examples of PCA tests in the skins of rats using A. sera from control mice lacking 2,4-D-specific IgE, B. sera containing 2,4-D-specific IgE, C. sera containing MMA-specific IgE, and D. sera containing DNP-specific IgE.

The positive control group produced anti-DNP IgE antibodies at all time intervals examined (Figures 7 and 8). Titers were considerably elevated following the second immunization.

Sera from the mice that received aluminum hydroxide and saline alone produced no positive reactions when tested in rats challenged with 2,4-D-BSA or DNP-BSA.

The sera taken on days 8, 15, 22, and 29 from mice that received epicutaneous applications of 2,4-D contained no measureable specific IgE. However, DNFB-treated mice had detectable DNP-specific IgE levels on days 22 and 29. The sera of saline-pretreated and cyclophosphamide-pretreated mice treated with DNFB had respective geometric mean titers and standard errors of 10.1 $\pm$ 1.02 (30% responding) and 8.0 $\pm$ 0.0 (60% responding) on day 21 and 12.1 $\pm$ 1.03 (50% responding) and 12.7 $\pm$ 1.10 (90% responding) on day 28.

Measurement of 2,4-D-specific IgE antibodies using the PRAST procedure

The time periods required for the incubation of the serum sample with the 2,4-D-BSA-coated disk and the incubation of the ^{125}I-anti-IgE antibodies with the disk were determined using pooled sera collected at sacrifice on day 43 from mice sensitized with 1 ug of 2,4-D-KLH. The pooled sera had a PCA titer of 32. When the time of the first incubation with 2,4-D-specific serum was varied, the activity bound per disk increased during the first six hours and then remained constant to the twenty-fourth hour. Values for control serum remained fairly constant over the time

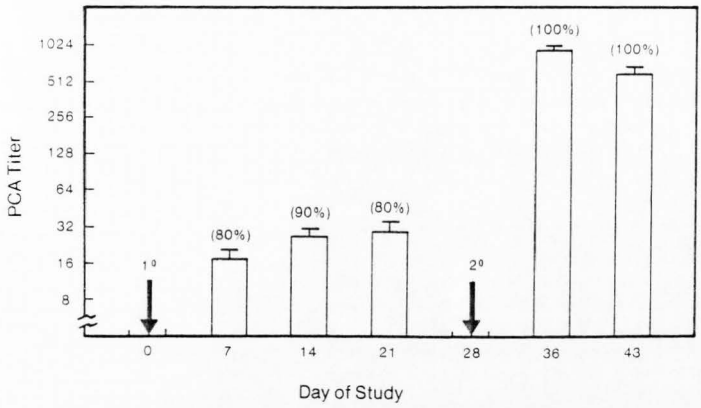


Figure 8. IgE antibody response specific for DNP in female BALB/c mice administered 1 ug of DNP-KLH i.p. at primary (1°) and seconday (2°) immunizations. The bars represent the geometric mean ± the standard error of the PCA titer for mice producing detectable circulating IgE antibodies. The percentage of mice that produced detectable antibodies is shown at the top of each bar.

period examined (Figure 9). The remainder of the PRAST analyses were performed using 18 hours for the first incubation.

With the second incubation with ^{125}I-anti-IgE, the activity bound continued to gradually increase as the time period was increased from 1 to 24 hours (Figure 10). Twenty-four hours was selected as the time of incubation for the remaining analyses.

Two-fold serial dilutions of the pool of 2,4-D-specific sera were analyzed to estimate the sensitivity of the PRAST procedure (Figure 11). Although the number of samples analyzed did not permit a statistical comparison, it appeared that the 2,4-D-specific serum could be distinguished from control serum on the basis of the cpm bound to the disks at a dilution of 1:32 and perhaps at 1:64. These results indicated that the PCA and PRAST titers were very similar, with the PRAST assay perhaps slightly more sensitive.

Thirteen serum samples with predetermined PCA titers taken from mice that received 1 ug or 100 ug of 2,4-D-KLH at day 43 were analyzed by PRAST. The mean cpm bound to the disks for serum samples with PCA titers of ‹8 to 64 are shown in Figure 12. The mean values from PRAST showed a pattern consistent with the PCA titers; however, the individual PRAST values for serum samples with the same PCA titer were variable, as shown in Table 3. Consequently, the R^2 is 28.5 percent. The PRAST results from samples diluted four-fold and the corresponding titers are presented in Figure 13.

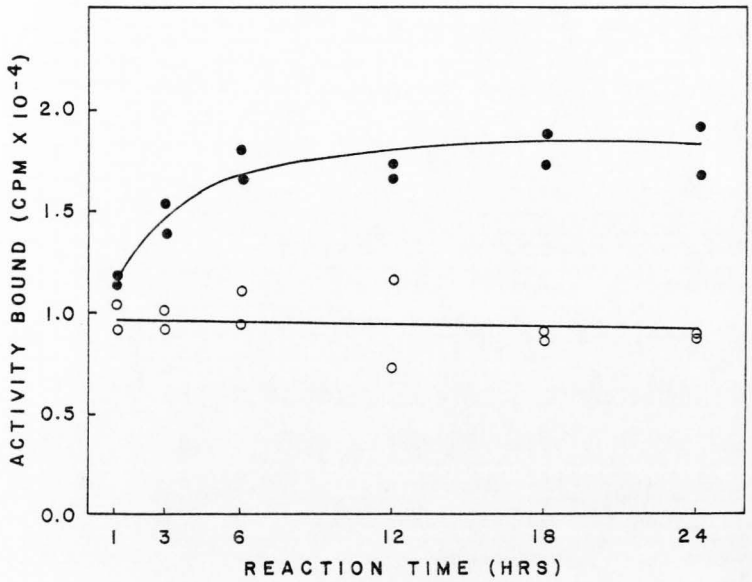


Figure 9. PRAST for 2,4-D-specific IgE with the reaction time of pooled sera with the antigen-bound disks varied and the reaction time of the disk with ^{125}I-anti-IgE antibodies (1.9 x 10^6 cpm/o.2 ml) constant at 18 hours. Pooled sera with PCA titer of 32, diluted 1:4 (●); pooled control sera, diluted 1:4 (○).

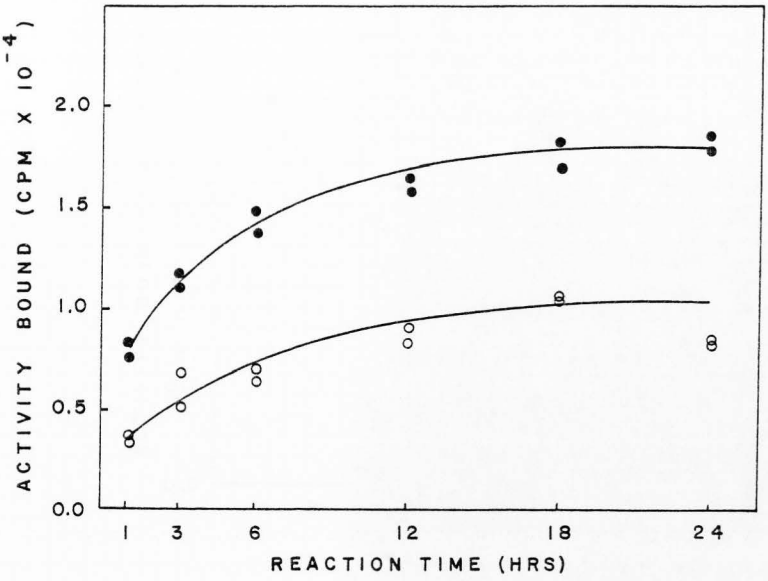


Figure 10. PRAST for 2,4-D-specific IgE with the reaction time of pooled sera with the antigen-bound disks constant at 18 hours and the reaction time of the disk with ^{125}I-anti-IgE antibodies (1.9 x 10^6 cpm/0.2 ml) varied. Pooled sera with PCA titer of 32, diluted 1:4 (●); pooled control sera, diluted 1:4 (○).

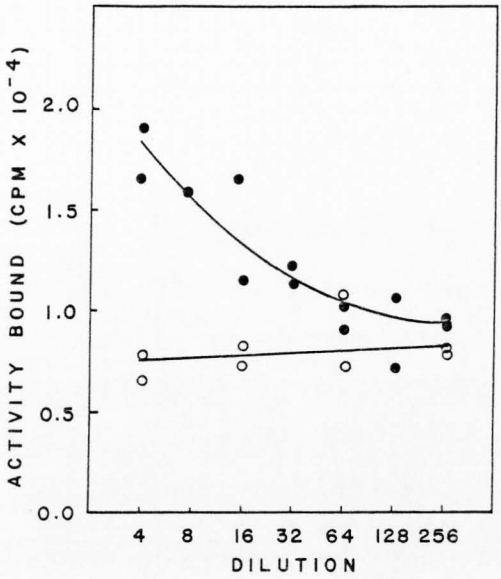


Figure 11. Sensitivity of the PRAST for 2,4-D-specific IgE of pooled sera with a PCA titer of 1:32 (●) and pooled control sera (o). ^{125}I-anti-IgE contained 1.9 x 10^6 cpm/0.2 ml.

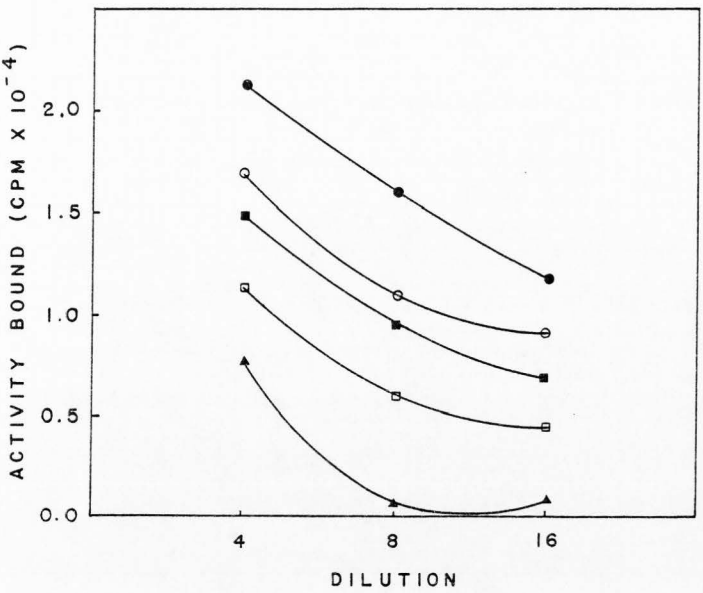


Figure 12. PRAST for 2,4-D-specific IgE of samples with predetermined PCA titers of 1:64 (●), 32 (○), 16 (■), 8 (□), and <8 (▲). Each point represents the mean of three serum samples analyzed in duplicate at a dilution of 1:4, with the exception of the points for the PCA titer of 64 which represents one serum sample. Values are corrected for activity bound to disks using control sera at a dilution of 1:4. ^{125}I-anti-IgE contained 1.9 x 10^6 cpm/0.2 ml.

Table 3. Mean and range of activity bound during PRAST analysis of serum samples with predetermined PCA titers. Each sample was analyzed in duplicate. Values are corrected for activity bound to disks using control sera at a dilution of 1:4. ^{125}I-anti-IgE contained 1.9 x 10^6 cpm/0.2 ml.

PCA Titer	No. of Mice	Dilution	Activity Bound (cpm x 10^{-4}) Mean	Range
64	1	4	2.09	2.05 - 2.13
		8	1.64	1.59 - 1.70
		16	1.18	1.14 - 1.21
32	3	4	1.68	0.92 - 3.88
		8	1.08	0.66 - 1.56
		16	0.91	0.43 - 1.03
16	3	4	1.48	0.72 - 2.24
		8	0.93	0.59 - 1.47
		16	0.69	0.42 - 0.90
8	3	4	1.13	0.75 - 1.42
		8	0.59	0.46 - 1.11
		16	0.46	0.37 - 0.56
‹8	3	4	0.76	0.00 - 1.33
		8	0.05	0.00 - 0.21
		16	0.10	0.00 - 0.26

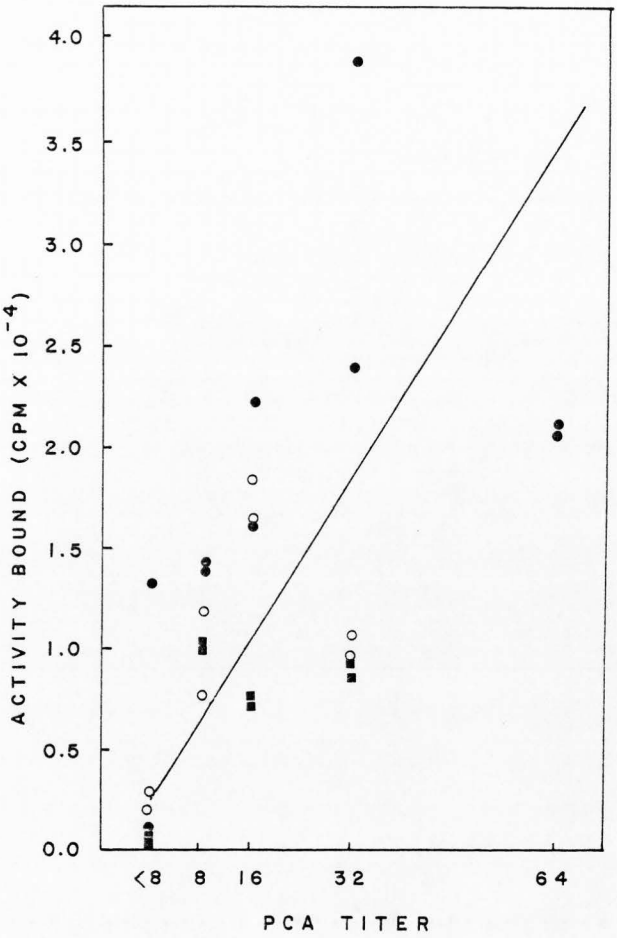


Figure 13. Comparison of the results of the PRAST and PCA tests. Samples analyzed by PRAST were diluted 1:4. Identical symbols at each PCA titer represent duplicate analyses. Values are corrected for activity bound to disks using control serum. ^{125}I-anti-IgE contained 1.9×10^6 cpm/0.2 ml.

Studies of DTH responses to 2,4-D and DNFB

2,4-D did not elicit a delayed-type hypersensitivity response when administered on either two consecutive days or weekly over a period of one month (Figures 14, 15, and 16). Neither ear thickness nor incorporation of [^{125}I]-UdR in the ear were different from control values. Similarly, the histology of the challenged ears of 2,4-D-treated mice did not differ from solvent-treated mice (Figures 17 and 18). In contrast, the DNFB-challenged ears of the mice sensitized with DNFB were thickened and contained an increased amount of [^{125}I]-UdR as compared to solvent-challenged ears of the same mice and the challenged ears of solvent-treated mice. Upon histologic examination, ears of DNFB-treated mice showed both lymphocyte infiltration and edema (Figure 19). The numbers of mast cells and basophils stained by toluidine blue appeared to be similar in the ears taken from sensitized and unsensitized mice.

Evaluation of Allergic Hypersensitivity to Malathion

Preparation of MMA-protein conjugates

Shown in Table 2 are the degrees of conjugation achieved with the MMA hapten coupled to KLH and BSA. The degrees of conjugation were considered near the optimum for elicitation and testing of MMA-specific IgE.

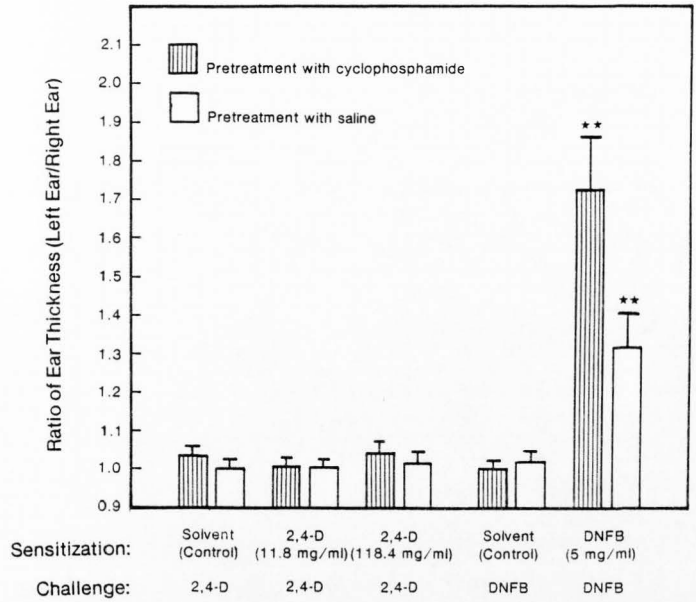


Figure 14. Delayed-type hypersensitvity as measured by increase in ear thickness following epicutaneous sensitization with 2,4-D or DNFB on two consecutive days. Left ear was challenged with the test compound, right ear with solvent. The bars represent the mean $\pm$ the standard error from 8 female BALB/c mice. Statistical significance according to the t test for unequal variances is indicated by **, $p<0.01$.

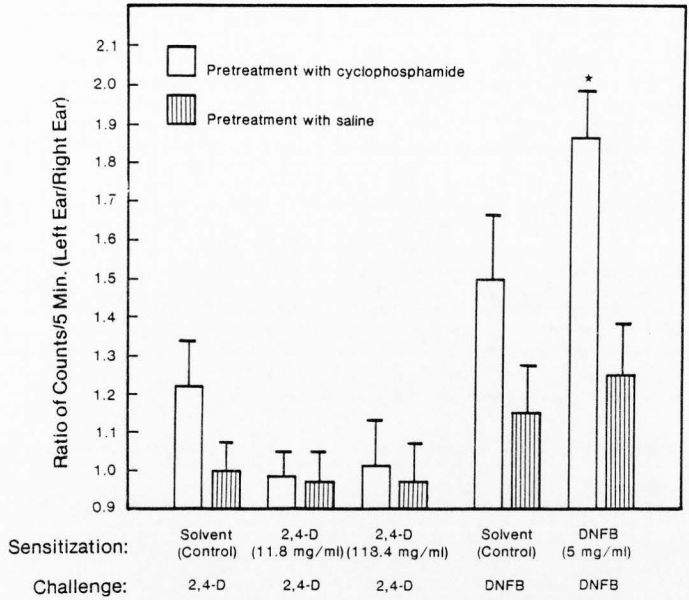


Figure 15. Delayed-type hypersensitivity as measured by incorporation of 5-[^{125}I]iodo-2'deoxyuridine following epicutaneous sensitization with 2,4-D or DNFB on two consecutive days. Left ear was challenged with the test compound, right ear with solvent. The bars represent the mean $\pm$ the standard error from 8 female BALB/c mice. Statistical significance according to the t test for equal variances is indicated by *, $p<0.05$.

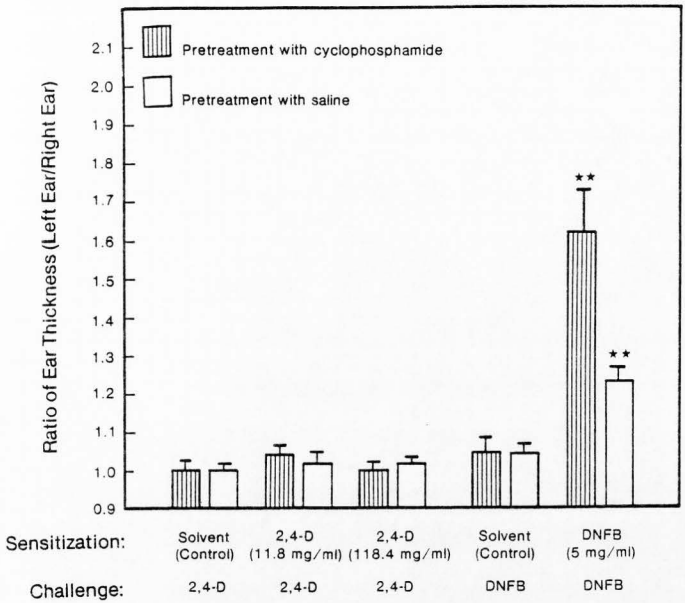


Figure 16. Delayed-type hypersensitivity as measured by increase in ear thickness following epicutaneous sensitizations with 2,4-D or DNFB over four weeks. Left ear was challenged with the test compound, right ear with solvent. The bars represent the mean $\pm$ the standard error from 10 2,4-D- or DNFB-treated or 6 solvent-treated female BALB/c mice. Statistical significance according to the t test for unequal variances is indicated by **, $p<0.01$.

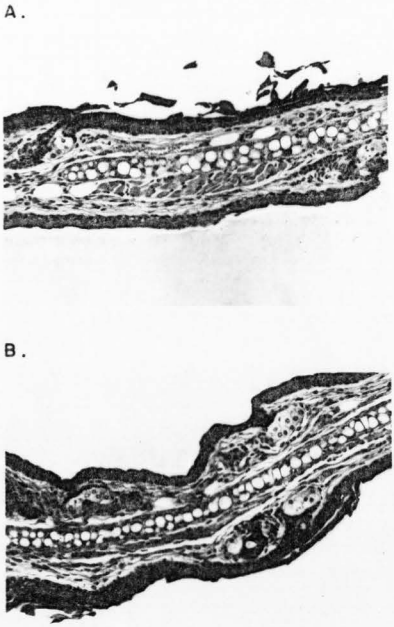


Figure 17. Histologic sections of ears (H & E stain, x 160) from mice pretreated with cyclophosphamide, sensitized with solvent, and challenged with A. 2,4-D (11.8 mg/ml) or B. solvent.

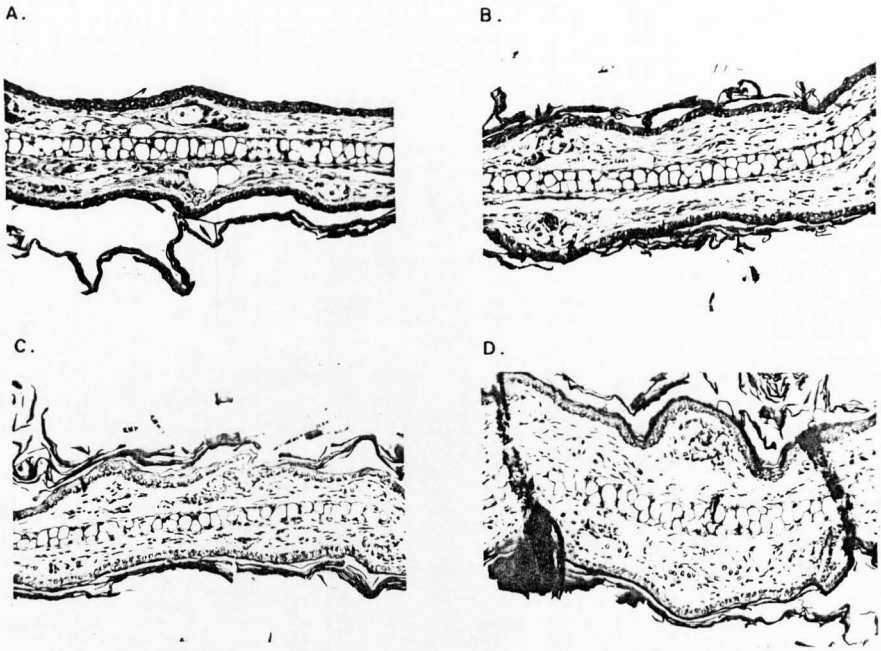


Figure 18. Histological sections of ears (H & E stain, x 160) from mice pretreated with cyclophosphamide, sensitized with 2,4-D (118.4 mg/ml) and challenged with A. 2,4-D (11.8 mg/ml) or B. solvent, or sensitized with malathion 37.4 mg/ml) and challenged with C. malathion (17.8 mg/ml) or D. solvent.

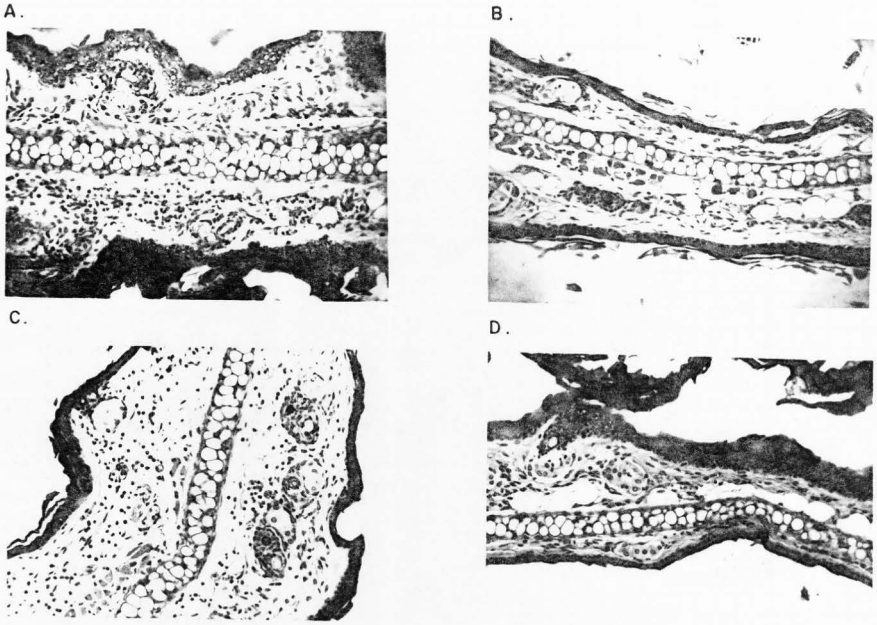


Figure 19. Histologic sections of ears (H & E stain, x 160) from mice pretreated with cyclophosphamide, sensitized with captafol (37.4 mg/ml) and challenged with A. captafol (18.7 mg/ml) or B. solvent, or sensitized with DNFB (5 mg/ml) and challenged with C. DNFB (2 mg/ml) or D. solvent.

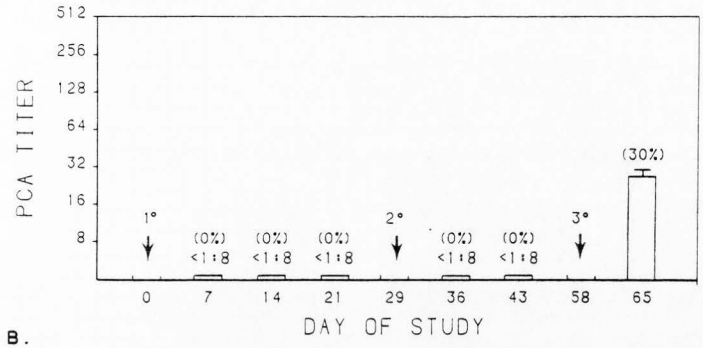
Measurement of MMA- and
DNP-specific IgE antibodies
using the PCA test

MMA-specific IgE antibodies were observed in mice following secondary immunizations with 10 and 100 ug of MMA-KLH and following tertiary immunization with all dose levels of MMA-KLH (Figures 7 and 20). In the group of mice that received 100 ug of conjugate per immunization, 90% of the mice produced detectable IgE following the secondary immunization. This response was boosted by a tertiary immunization, resulting in a higher geometric mean titer with all mice producing detectable IgE. Only one of ten mice that received 10 ug of MMA-KLH produced measureable IgE following a secondary immunization. Upon tertiary immunization, however, all of the mice responded and produced a mean titer equivalent to the group that received 100 ug of conjugate. The dose of 1 ug of MMA-KLH produced low titers in only 30% of the mice following three immunizations.

The group of mice that received the positive control conjugate, DNP-KLH, produced anti-DNP IgE antibodies at all time intervals examined (Figure 21). The mean titers were boosted considerably by the second immunization and only slightly by the third immunization.

Sera from the mice that were immunized with aluminum hydroxide and saline alone produced no measureable specific IgE responses when tested in rats challenged with MMA-BSA or DNP-BSA.

A.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DAY OF STUDY

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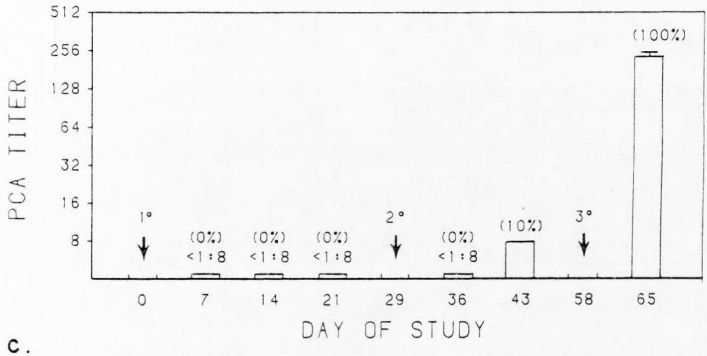
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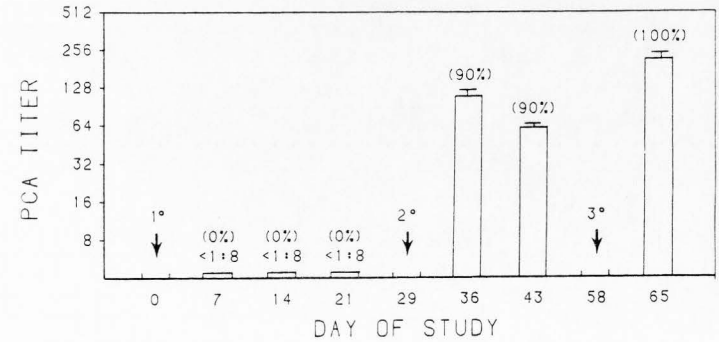
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DAY OF STUDY

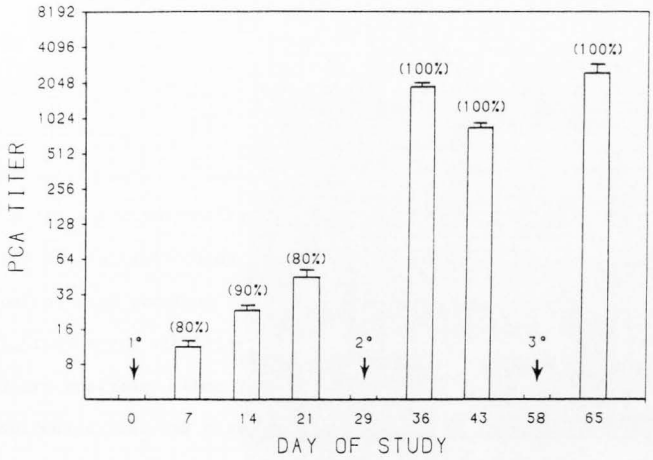

Figure 21. IgE antibody response for DNP in female BALB/c mice adminstered 1 ug of DNP-KLH i.p. at primary (1°), secondary (2°), and tertiary (3°) immunizations. The bars represent the geometric mean ± the standard error and the numbers in brackets the percentage of mice producing detectable circulating IgE antibodies.

No MMA-specific IgE could be detected in sera taken at days 8, 15, 22, and 29 from mice dosed epicutaneously with malathion following pretreatment with cyclophosphamide or saline.

Measurement of MMA-specific IgE antibodies using the PRAST procedure

Pooled sera collected at death from the mice that received 100 ug of MMA-KLH were used to determine the optimum time periods for the incubation of the sample with the conjugate-coated disk and incubation of the ^{125}I-anti-IgE antibodies with the disk. The pooled sera had a PCA titer of 256. The activity bound per disk reached a maximum following six hours of incubation of the sample with the antigen-coated disk (Figure 22). Values obtained using control sera remained constant over the time interval examined. Eighteen hours was selected as the time period to be used for future analyses. When the time of reaction of the ^{125}I-anti-IgE antibodies with the disk was varied, no maximum activity bound to the disks was reached using sera containing MMA-specific IgE or control sera (Figure 23). The remainder of the serum samples was tested using 24 hours for the second incubation.

The same pool of MMA-specific sera was used to determine the sensitivity of the PRAST procedure (Figure 24). The values for the sera containing MMA-specific IgE appear to be distinguished from control values at a dilution of 1:512, a two-fold higher dilution than the PCA titer for the pooled sera.

The results of the PRAST analysis on six serum samples of predetermined PCA titers from 32 to 1024 taken at day 65 from mice

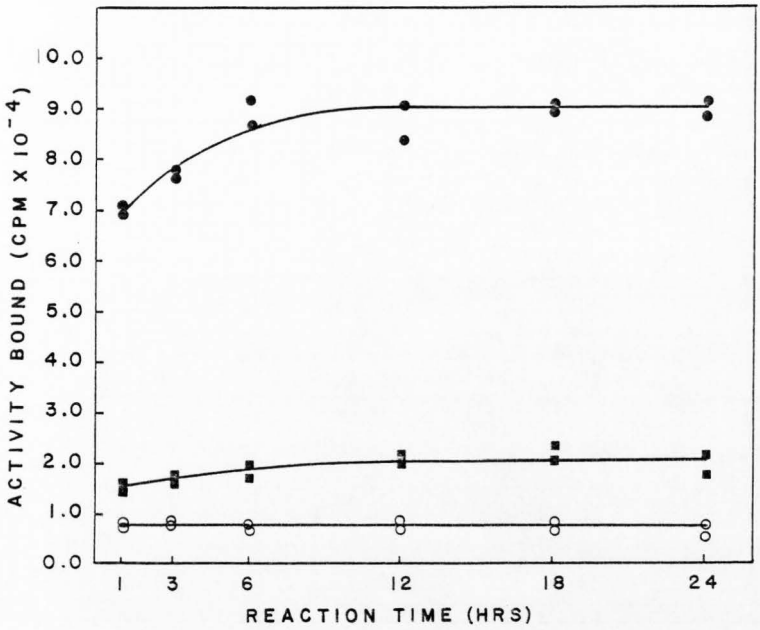

Figure 22. PRAST for MMA-specific IgE with the reaction time of pooled sera with the antigen-bound disks varied and the reaction time of the disk with ^{125}I-anti-IgE antibodies (8.8 x 10^5 cpm/0.2 ml) constant at 18 hours. Pooled sera with a PCA titer of 256, diluted 1:4 (●) and 1:64 (■); pooled control sera diluted 1:4 (○).

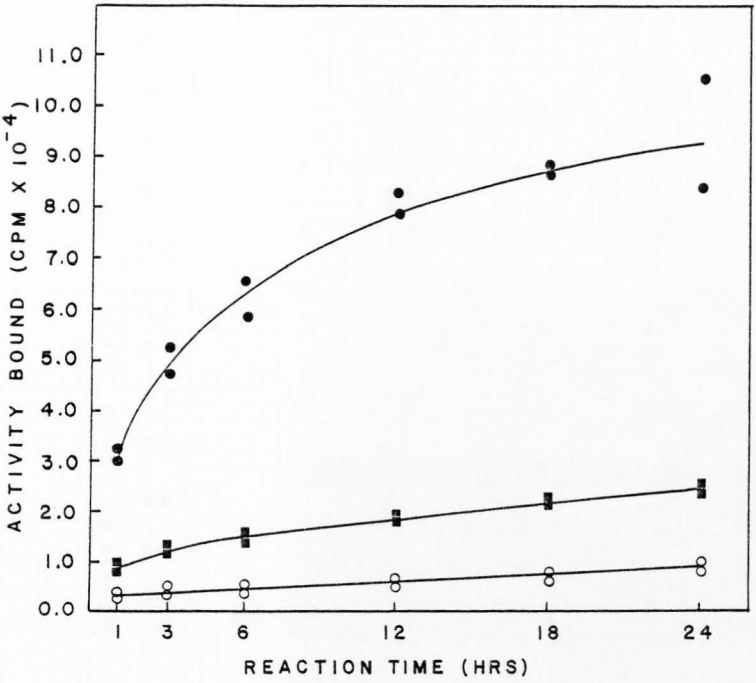

Figure 23. PRAST for MMA-specific IgE with the reaction time of pooled sera with the antigen-bound disks constant at 18 hours and the reaction time of the disk with ^{125}I-anti-IgE antibodies (8.8 x 10^5 cpm/0.1 ml) varied. Pooled sera with a PCA titer of 256, diluted 1:4 (●) and 1:64 (■); pooled control sera diluted 1:4 (○).

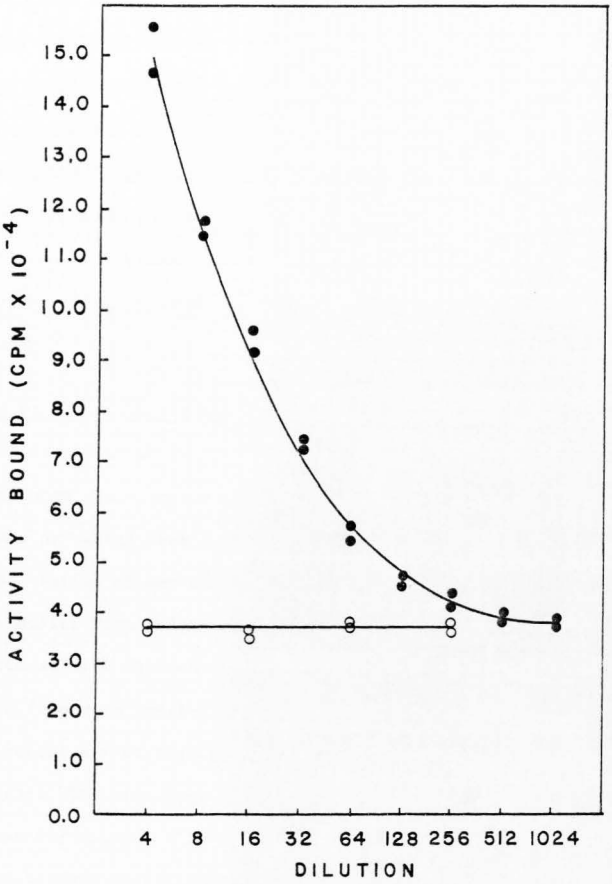

Figure 24. Sensitivity of the PRAST for MMA-specific IgE performed with pooled sera with a PCA titer of 1:256 (●) and pooled control sera (○). ^{125}I-anti-IgE contained 1.1 x 10^5 cpm/0.2 ml.

that received 10 ug of MMA-KLH are shown in Figure 25. The sera that gave PCA titers of 512 and 256 had almost identical values. Similar results were also obtained with the sera that gave PCA titers of 128 and 64. This variability is within the range observed for the PRAST for 2,4-D-specific IgE.

PRAST results for sera diluted four-fold and corresponding PCA titers are compared in Figure 26. The R^2 for the relationship between the PRAST and PCA results was 91.4 percent, indicating good predictability.

Studies of DTH responses to malathion

Malathion did not elicit a delayed-type hypersensitivity response when administered epicutaneously on either two consecutive days or weekly over a period of one month (Figures 27, 28, and 29). Ratios of ear thicknesses and incorporation of [^{125}I]-UdR did not differ from values obtained from solvent-treated mice. Pretreatment with cyclophosphamide did not alter these results. The data from mice treated with DNFB shown in Figures 27, 28, and 29 are repeated from Figures 14, 15, and 16 for purposes of comparison. The histology of ears from mice treated with malathion and solvent also did not differ (Figure 17 and 18). Sections stained with toluidine blue showed similar numbers of mast cells and basophils in ears of malathion- and solvent-treated mice.

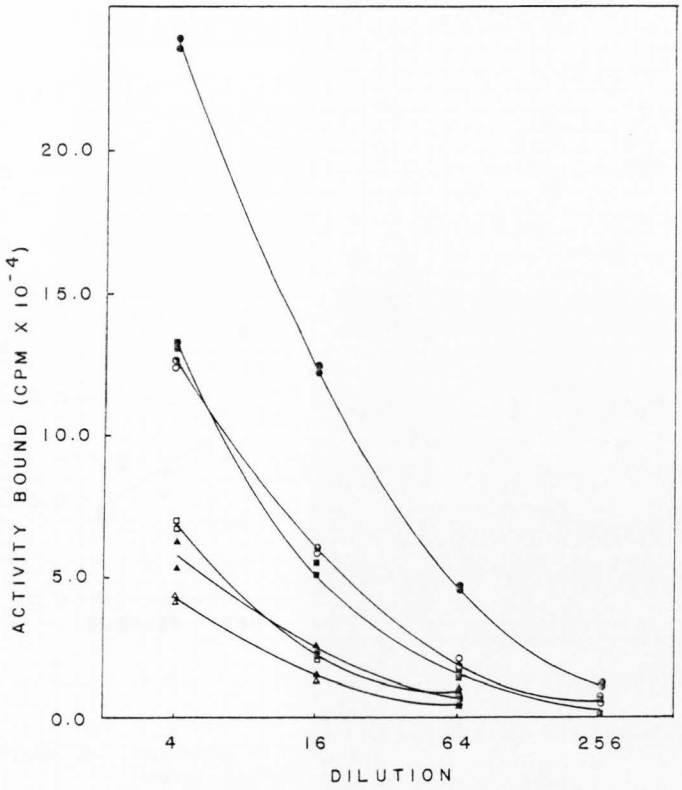

Figure 25. PRAST for MMA-specific IgE of samples with predetermined PCA titers of 1:1024 (●), 512 (○), 256 (■), 128 (□), 64 (▲), and 32 (△). One serum sample with each PCA titer was analyzed in duplicate at a dilution of 1:4. Values are corrected for activity bound to disks using control sera at a dilution at 1:4. ^{125}I-anti-IgE contained 1.1 x 10^6 cpm/0.2 ml.

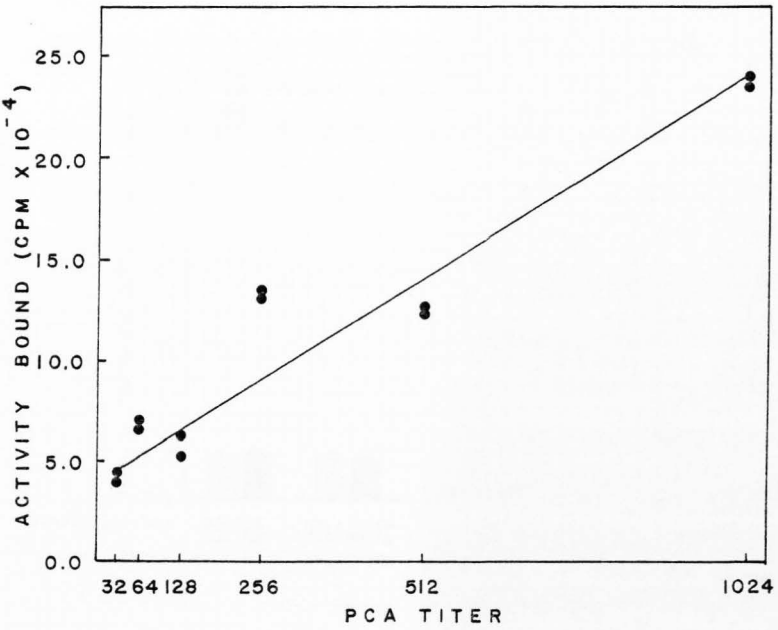

Figure 26. Comparison of the results of the PRAST and PCA tests. Samples analyzed by PRAST were diluted 1:4. Values are corrected for activity bound to disks using control sera. ^{125}I-anti-IgE contained 1.9 x 10^6 cpm/0.2 ml.

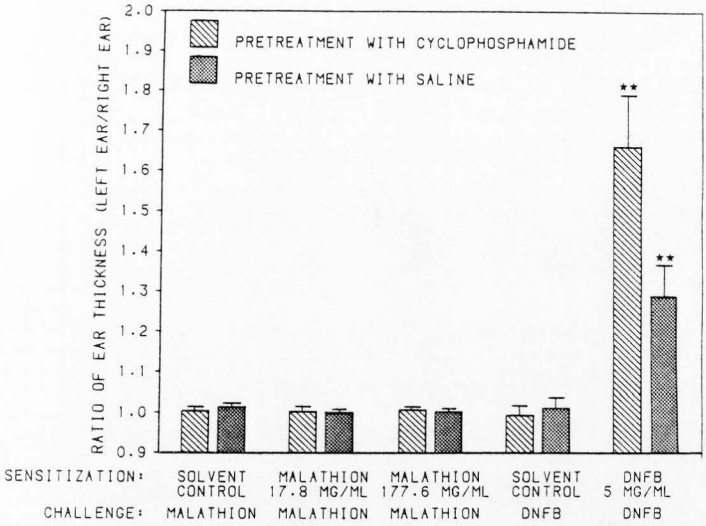

Figure 27. Delayed-type hypersensitivity as measured by increase in ear thickness following epicutaneous sensitization with malathion or DNFB on two consecutive days. Left ear was challenged with the test compound, right ear was challenged with solvent. The bars represent the mean $\pm$ standard error from 8 female BALB/c mice. Statistical significance according to the t test for unequal variances is indicated by **, $p<0.01$.

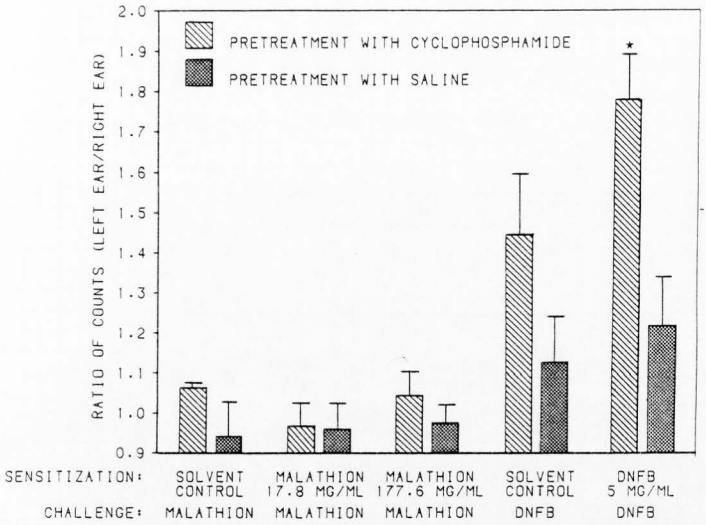

Figure 28. Delayed-type hypersensitivity as measured by incorporation of 5-[^{125}I]iodo-2'deoxyuridine following epicutaneous sensitization with malathion or DNFB on two consecutive days. Left ear was challenged with the test compound, right ear was challenged with solvent. The bars represent the mean $\pm$ the standard error from 8 female BALB/c mice. Statistical significance according to the t test for equal variances is indicated by *, $p<0.05$.

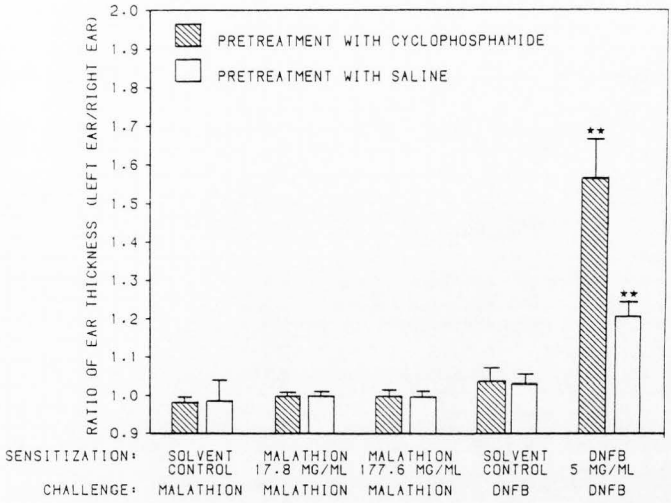

Figure 29. Delayed-type hypersensitivity as measured by increase in ear thickness following epicutaneous sensitizations with malathion or DNFB over four weeks. Left ear was challenged with the test compound, right ear was challenged with solvent. The bars represent the mean $\pm$ the standard error from 10 malathion- or DNFB-treated or 6 solvent-treated female BALB/c mice. Statistical significance according to the t test for unequal variances is indicated by **, $p<0.01$.

Evaluation of Allergic Hypersensitivity to Captafol

Studies of DTH responses to captafol

Captafol at 18.7 and 37.4 mg/ml produced DTH responses in mice at both dose levels. Following two daily epicutaneous applications, ratios of ear thicknesses and incorporation of [^{125}I]-UdR were significantly elevated in comparison to values for control mice treated with solvent (Figures 30 and 31). Mice pretreated with cyclophosphamide responded to a greater extent than mice pretreated with saline. Mice sensitized with 18.7 mg/ml gave higher responses than mice sensitized with 37.4 mg/ml. Results obtained from mice challenged with DNFB are repeated in Figures 30 and 31 from Figures 14 and 17 for purposes of comparison. Histologic sections of ears from captafol-treated mice revealed thickening with edema and cellular infiltration (Figure 19). The number of mast cells and basophils stained by toluidine blue did not appear to differ between ears of sensitized and solvent-treated mice.

Multiple sensitizations with captafol over the period of a month also produced delayed-type hypersensitivity as measured by increases in ear thicknesses (Figures 32 and 33). The lower dose level of captafol again produced larger responses than the higher dose level. In mice pretreated with saline, the ratio of ear thicknesses increased from 24 to 48 hours after challenge in mice sensitized and challenged with captafol. Mice sensitized and challenged with DNFB showed a decrease in ratio of ear thicknesses

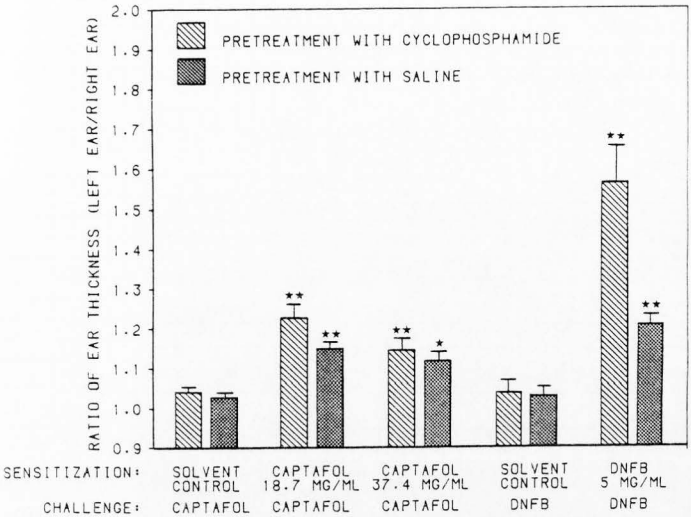

Figure 30. Delayed-type hypersensitivity as measured by increase in ear thickness following epicutaneous sensitization with captafol or DNFB on two consecutive days. Left ear was challenged with the test compound, right ear was challenged with solvent. The bars represent the mean $\pm$ the standard error from 8 female BALB/c mice. Statistical significance according to the t test for equal variances for saline-pretreated mice that received captafol at the low dose and according to the t test for unequal variances for other groups is indicated by *, $p<0.05$ or **, $p<0.01$.

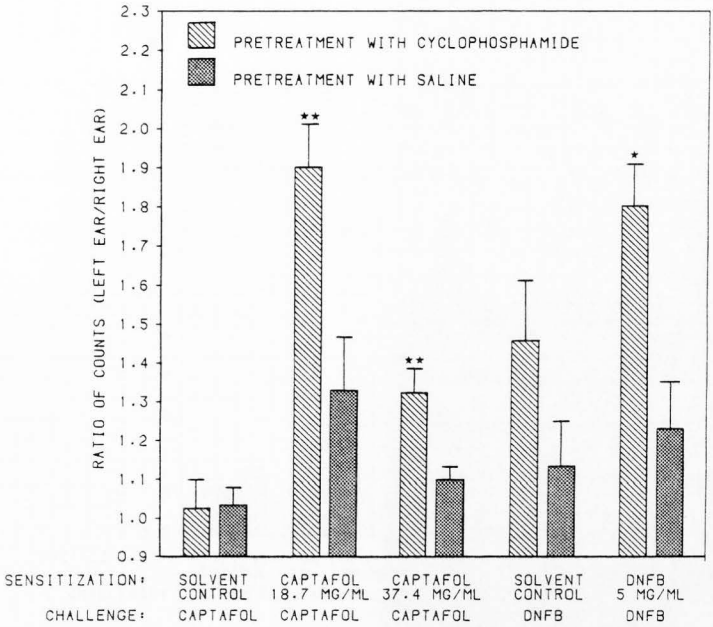

Figure 31. Delayed-type hypersensitivity as measured by incorporation of 5-[^{125}I]iodo-2'-deoxyuridine following epicutaneous sensitization with captafol or DNFB on two consecutive days. Left ear was challenged with the test compound, right ear was challenged with solvent. The bars represent the mean $\pm$ the standard error from 8 female BALB/c mice. Statistical significance according to the t test for equal variances is indicated by *, $p<0.05$ or **, $p<0.01$.

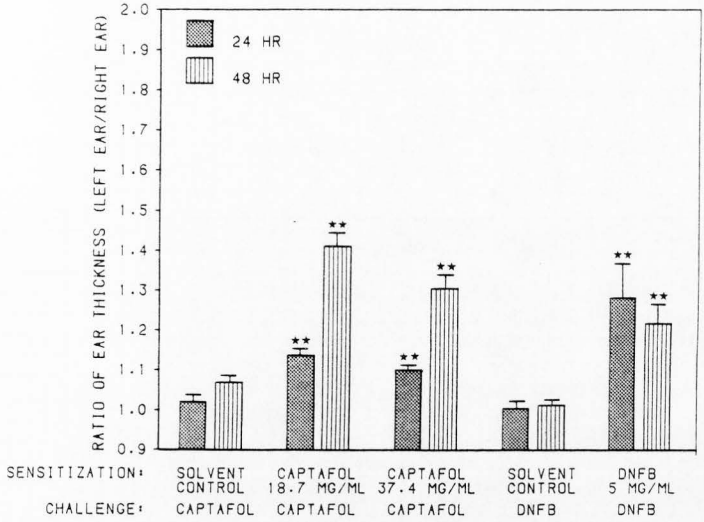

Figure 32. Delayed-type hypersensitivity as measured by increase in ear thickness following pretreatment with saline and epicutaneous sensitizations with captafol or DNFB over four weeks. Left ear was challenged with the test compound, right ear was challenged with solvent. The bars represent the mean $\pm$ the standard error from 10 captafol- or DNFB-treated or 6 solvent-treated female BALB/c mice. Statistical significnace according to the t test for equal variances for captafol-treated mice at 24 hrs and according to the t test for unequal variances for other groups is indicated by **, $p<0.01$.

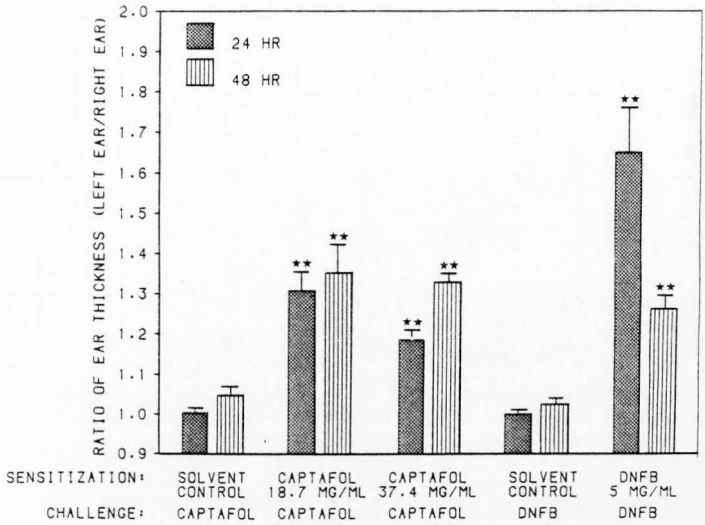

Figure 33. Delayed-type hypersensitivity as measured by increase in ear thickness following pretreatment with cyclophosphamide and epicutaneous sensitizations with captafol or DNFB over four weeks. Left ear was challenged with the test compound, right ear was challenged with solvent. The bars represent the mean $\pm$ the standard error from 10 captafol- or DNFB-treated or 6 solvent-treated female BALB/c mice. Statistical significance according to the t test for unequal variances is indicated by **, $p<0.01$.

(Figure 32). Cyclophosphamide-pretreated mice demonstrated similar changes in ear thickness with time (Figure 33). In captafol-treated mice, pretreatment with cyclophosphamide increased the response at 24 hours but did not significantly alter the maximum response measured at 48 hours as compared to saline-pretreated mice. In contrast, the ratio of ear thicknesses was greatly elevated at 24 hours and only slightly changed at 48 hours in mice treated with DNFB and pretreated with cyclophosphamide as compared to those pretreated with saline.

Control mice treated with solvent showed a slight, statistically insignificant increase in thickness of ears challenged with captafol (Figures 30, 32, and 33) and DNFB (Figure 33). This response can be attributed to the slight acute irritancy of these chemicals at the doses administered.

DISCUSSION

This study investigated the sensitizing potential in BALB/c mice of three pesticides implicated in reports of hypersensitivity involving dermatitis and respiratory complaints in exposed persons. The abilities of 2,4-D, malathion, captafol, and a known sensitizer, DNFB, to produce DTH were studied using epicutaneous applications of the chemicals. IgE-mediated hypersensitivity to 2,4-D, malathion, and DNFB was studied using epicutaneous applications of the chemicals and using intraperitoneal injections of the pesticides and DNP coupled to a protein to simulate an allergenic conjugate that might form upon coupling to body proteins in the exposed human. IgE antibodies specific for 2,4-D, MMA, and DNP were elicited by sensitizations with 2,4-D-KLH, MMA-KLH, and DNP-KLH, respectively. Epicutaneous applications of 2,4-D and malathion did not produce either DTH or detectable circulating specific IgE. DTH to captafol and DNFB was elicited by epicutaneous sensitizations.

Pesticide-protein Conjugates

In the pesticide-protein conjugates, the carboxylic groups of 2,4-D and the diacid metabolite of malathion were bound to available lysine amino groups in KLH and BSA by formation of peptide bonds. The conjugates of 2,4-D and protein were formed using the coupling agents EEDQ and EDC whereas the conjugates with

the metabolite of malathion relied on the reactivity of the anhydride derivative.

The dichlorophenoxy group of 2,4-D was expected to protrude furthest from the surface of the conjugated protein and thus remain available as the primary antigenic determinant of the hapten. This structure is common to all formulations of 2,4-D and would be the available determinant following most probable coupling that might occur with body proteins following metabolism of the amine or ester portion of these formulations. Coupling to 2,4-D at the ring, such as by the coupling of the diazonium salt formed from 2,4-dichloro-5-aminophenoxyacetic acid (Rinder and Fleeker, 1981), would have produced a conjugate with a different antigenic structure. The conjugate prepared by Rinder and Fleeker has been shown to elicit antibodies that bind 2,4-D and 2,4,5-T but has not been tested specifically for its ability to elicit IgE.

In the MMA conjugates, the dimethoxyphosphorodithio portion of the hapten formed the available antigenic structure. Again, this conjugate could be formed with body proteins following hydrolysis of the carboxyester linkage in malathion which is a major detoxification pathway of malathion in mammals (Krueger and O'Brien, 1959). It is possible that residual maleic anhydride or O,O-dimethylphosphorodithioate in the MMA preparation could have formed conjugates also. However, these compounds would not have been present at high concentrations. In addition, a search of the

published literature revealed no evidence that either of these compounds are hypersensitizing chemicals. Since the MMA conjugates were not analyzed for purity of the haptens, the possibility of other haptens beside MMA present in the conjugates can not be completely excluded.

The density of haptenic groups in conjugates has been shown to influence the antibody response. Either too few or too many haptens produce no response or a brief response which cannot be boosted (Klause and Cross, 1974; Quijada et al., 1974). Erlanger (1980) noted that his group has never failed to obtain a response with conjugates having as few as two haptenic groups per protein molecule, although a longer period of sensitization may be required to obtain equivalent antibody titers. The degree of conjugation of the 2,4-D-KLH used for sensitization was five 2,4-D molecules per 100,000 daltons of KLH. The relatively low titers of 2,4-D-specific IgE elicited upon secondary immunization may be explained in part by the low epitope density of the 2,4-D conjugate. Tertiary and quartenary sensitizations would be expected to boost the response. Following the results obtained with two immunizations of 2,4-D-KLH, a third immunization was added to the design of the study of MMA-KLH. The degrees of conjugation of the MMA-KLH and DNP-KLH (9.0 and 10.6, respectively) were considered optimum for elicitation of antibodies.

IgE Antibody Response to 2,4-D, MMA, and DNP

Based on the results of PCA analyses, 2,4-D-KLH, MMA-KLH, and DNP-KLH all elicited hapten-specific IgE antibodies, but with differing patterns of response.

The lowest dose of 2,4-D-KLH, 1 ug, elicited the highest titers with the highest proportion of mice responding following two immunizations as compared to the 10 and 100 ug doses. This pattern of response was reported first by Levine and Vaz (1970) who noted that high doses of benzylpenicilloyl and DNP conjugates elicited little or no IgE antibody production while eliciting good levels of IgG antibody. Tamura and Ishizaka (1978) showed that immunization with relatively large doses of ovalbumin resulted in the generation of antigen-specific suppressor T lymphocytes which were not demonstrated in mice that were immunized with small doses. These researchers concluded that the IgE response in relation to dose depended on the elicitation of suppressor T lymphocytes and not solely on the activity of helper T lymphocytes or B memory cells. Other studies in mice and rats indicated that the size of the primary dose of antigen is important in determining the primary response itself (Hamaoka et al., 1974; Jarrett, 1978; Lee and Sehon, 1978). In this study, it appeared that the 10 and 100 ug doses were unable to elicit a primary IgE response but did generate a dose-related suppression of the secondary response both in terms of a decrease in the percentage of mice with detectable specific IgE and a decrease in the mean titers observed. The one mouse that responded to the 100 ug dose

apparently lacked the degree of suppression observed in the rest of the group.

In contrast to the results produced with 2,4-D-KLH, the highest titers of MMA-specific IgE were measured after two immunizations in mice that received 100 ug and after three immunizations in mice that received 10 or 100 ug of MMA-KLH. The lowest dose, 1 ug, elicited a response at relatively low titers in only 30% of the mice following three immunizations. These results indicate that a larger number of MMA molecules must be present or repeated small doses must be administered for a specific IgE antibody response to be triggered as compared to 2,4-D and DNP groups. This requirement for a relatively large single dose of malathion for the generation of an IgE response is similar to the findings reported by Milby and Epstein (1964) regarding sensitization in humans. These researchers found that the sensitizing potential of malathion at a concentration of 10% in alcohol was much stronger than that of 1% and 0.1% solutions. The sensitizing potential of single large doses of MMA-protein conjugate is also reflected in the ability of Vijay et al. (1978) to elicit measureable IgE titers using the relatively enormous doses of 1 and 2 mg of MMA-BSA in rats.

Once initiated, the MMA-specific response was greater than that produced by 2,4-D-KLH but less than that of DNP-KLH, as can be seen in a comparison of the PCA titers at day 36 following the second immunization. Thus, within the range of doses studied, the

three haptens can be ranked in order of increasing sensitizing potential as follows: 2,4-D, malathion, and DNFB.

Epicutaneous applications of 2,4-D and malathion did not produce detectable circulating specific IgE. Pretreatment with cyclophosphamide did not alter these results, although cyclophosphamide at the dose level used in this study has been shown to enhance the IgE response to DNP-KLH and DNP conjugated to Ascaris proteins administered intraperitoneally (Chiroazzi et al., 1976, 1977) by reduction of suppressor T lymphocytes. However, DNP-specific IgE was measured at low titers similar to those reported by Thomas et al. (1976, 1978a, 1978b) for oxazolone and picryl chloride and probably similar, also, to the "feebly detectable" antibodies to dinitrochlorobenzene reported by Chase (1947). Cyclophosphamide pretreatment appeared to increase the percentage of mice producing detectable IgE toward DNP but not the titers.

Comparison of PRAST and PCA Procedures

The PRAST assays developed for IgE specific to 2,4-D and MMA were successful in quantifying antibody levels in sera previously analyzed using the PCA test. In the PRAST assays for both 2,4-D and MMA using pooled sera, the PRAST appeared to give results with IgE-containing sera which could be distinguished from control sera at the same or a two-fold higher dilution than the PCA titer. These results indicating that the PRAST is slightly more sensitive than the PCA test agree with those reported by Kelly et al. (1980) for monoclonal IgE antibody.

The variability observed in the results of the PRAST procedure for serum samples that gave identical PCA titers may be attributed to several differences between the two test procedures.

In the PRAST procedure, the amount of ^{125}I-anti-IgE bound to the paper disk depends in part on the concentration and affinity of the specific IgE in the serum sample for the antigen and the loss of bound specific IgE due to washing and during reaction with the ^{125}I-anti-IgE solution (Kelly et al., 1980). Only specific IgE with a minimum required affinity for the antigen would remain on the disk following the entire two-day test procedure. However, IgE with lesser affinities would be expected to bind to target cells in rat skin during the PCA test. This difference would produce different results between the two tests, depending on the heterogeneity and relative affinities of the IgE present in the samples.

In the PRAST procedure, competitive binding of non-IgE antibodies specific for the antigen could reduce the sensitivity of the test for specific IgE. Aluminum hydroxide was selected as the adjuvant used during sensitization to promote the elicitation of IgE rather than other antibody classes (Revoltella and Ovary, 1969). Nonetheless, any antigen-specific "blocking" antibodies present in the serum sample which bound to antigen on the disk would prevent antigen-specific IgE from binding, and thus reduce the labelled anti-IgE that could bind. As demonstrated by Mota and Wong (1969) and Konig et al. (1974), "blocking" IgG antibodies in mouse serum do not bind to rat mast cells and do not interfere

with the rat PCA test for IgE. "Blocking" antibodies were not quantitated in this study but may explain part of the variability observed using the PRAST procedure to analyze samples with identical PCA titers.

Further variability may be introduced by several factors present in the PCA test procedures that are absent from the PRAST assay. First, the initial dilution of serum in saline for the PCA test was assumed to be 1:8 based on a hematocrit of 50%. Variability would be introduced by a larger or smaller percentage of red blood cells. Each dilution of serum was tested in two rats and considered positive if a reaction of 5 mm or greater diameter was observed. It was apparent that some rats produced larger reactions than other rats when injected with the same sample. It is possible that some of the serum dilutions were tested in two rats that happened to be relatively non-reactive, thus yielding a lower PCA titer than possible in other more reactive rats. This question could have been resolved by testing a sample of known PCA titer in each rat to determine its relative reactivity. In addition, the properties of different regions of the skin on an animal are known to vary (Auerbach and Auerbach, 1982). Although different areas of the backs of the two rats were utilized for the two injections of the same sample, differences in reactivity of skin regions could have introduced additional variability into the results of the PCA tests.

Tests of DTH

As indicated by measurement of ear thickness, accumulation of [^{125}I]-UdR in ears, and histology of ears following challenge, neither 2,4-D nor malathion elicited DTH. Pretreatment of the mice with cyclophosphamide did not alter these results, although cyclophosphamide administered shortly before sensitization has been shown to enhance DTH responses by the decrease of suppressor T lymphocytes (Lagrange et al., 1974; Lagrange and Mackaness, 1975; Askenase et al., 1975; Asherson et al, 1979).

Malathion has been shown to be a sensitizer in guinea pigs using intradermal injections of 10% and 5% solutions, topical applications of 10% but not 25% solutions, and combined intradermal and topical administrations for sensitization (Magnusson and Kligman, 1970). The same report presented negative results using the Landsteiner-Draize procedure of multiple injections of a 0.1% solution. Thus, malathion appears to be a weak to moderate sensitizer that was detected in guinea pigs under specific test conditions but not in BALB/c mice with epicutaneous applications of 1.8% and 17.8% solutions.

Guinea pig sensitization test results with 2,4-D are not available in the published literature for comparison with results obtained in this study. However, the negative results using 2,4-D acid are not completely unexpected given the relatively poor absorption of this molecule through the lipophilic barrier presented by the skin (Feldmann and Maibach, 1974). The fat

solubility of other formulations, particularly the esters of 2,4-D, is much greater and would lead to increased absorption and perhaps increased sensitizing potential. These other formulations remain to be tested.

Captafol produced significant DTH responses in terms of all three parameters, as did the positive control chemical, DNFB. The smaller dose of captafol, 18.7 mg/ml, produced larger responses than the larger dose, 37.4 mg/ml. This dose response pattern has been attributed to the elicitation of increased suppressor cells by large doses of sensitizer (Sy et al., 1977).

Compared to pretreatment with saline, cyclophosphamide-pretreatment produced larger DTH responses when measured 24 hours after challenge following sensitization with either dose level of captafol and DNFB on two consecutive days or over the course of four weeks. The effect of cyclophosphamide pretreatment was greater in mice that received the lower dose level. Asherson et al. (1979) found little response to cyclophosphamide with mice treated with oxazolone at high levels whereas the response to lower doses was approximately doubled by cyclophosphamide to the level of response elicited by the higher dose level. Asherson et al. (1979) suggested that at high doses, effector cells respond to the antigen to a greater extent than suppressor cells, producing a substantial DTH response and that cyclophosphamide depression of suppressor cells is relatively insignificant. The effects of cyclophosphamide on suppressor cells would thus become more important at lower doses of antigen where the balance of effector

and suppressor systems is closer. However, in these studies with captafol, the suppressor cell population appeared to be more important in the high dose animals, resulting in a smaller response at the high dose that at the low dose. A wider range of dose levels of captafol and specific consideration of T lymphocyte subpopulations by syngeneic cell transfer studies would be required to explain the actions of dose levels and cyclophosphamide on effector and suppressor cell subpopulations.

In the mice with the longer sensitization period, ear thickness was measured at 48 hours as well as at 24 hours post-challenge. Ear thickness increased from 24 to 48 hours in mice sensitized with captafol and decreased in mice sensitized to DNFB. Pretreatment with cyclophosphamide boosted the response at 24 hours but not at 48 hours in captafol-treated mice. Thus, it appeared that the time course of the DTH response and recovery of regulatory mechanisms differed between captafol and DNFB.

The BALB/c Strain of Mice as a Model of Hypersensitivity

Within the limitations of the studies performed, it is possible to make an initial assessment of the BALB/c strain of mice as a model of both IgE-mediated and delayed-type hypersensitivity.

The mice were capable of mounting an IgE response to the pesticide-protein conjugates tested. The relative magnitudes of the IgE responses to 2,4-D, MMA, and DNP agreed with the relative immunogenicity of these compounds as indicated by reports of

hypersensitivity in humans. Limitations to the use of mice for investigating IgE-mediated hypersensitivity include the small amount of blood that can be taken and the need for repeated immunizations to elicit a high titer of IgE; however, the large number of mice that can be easily maintained and tested may compensate for these drawbacks.

DTH responses were elicited in the mice to two of the three chemicals tested which have been reported to produce DTH in guinea pigs. It is possible that sensitization of the mice by intradermal injection or by epicutaneous application of the chemicals to another area of skin would have produced sensitization to 2,4-D and/or malathion. In particular, sensitization of the anterior dorsal region has been recently shown to produce markedly elevated DTH responses as compared to the posterior ventral region used in these studies (Auerbach and Auerbach, 1982). As the DTH tests were conducted in this study, however, it appears that the BALB/c strain of mice can respond to moderate to strong sensitizers such as captafol and DNFB but not weak to moderate sensitizers such as malathion.

Suggested Areas for Future Investigations

These studies have suggested several areas of additional research that would contribute to the understanding of allergic hypersensitivity to pesticides and that would exploit the procedures developed in this project.

First, PRAST assays for 2,4-D- and MMA-specific IgE could be very easily modified to measure IgE in human sera from individuals exposed to 2,4-D or malathion. The only change required would be the substitution of ^{125}I-labelled anti-human IgE antibodies for the ^{125}I-labelled anti-mouse IgE antibodies. Use of the PRAST procedure would permit the rapid detection of pesticide-specific IgE in humans claiming pesticide-related allergies due to occupational or accidental exposure. This information would be a valuable addition to the information produced by patch testing in the assessment of allergy.

Related to the question of human allergic responses is the likelihood of cross-reaction of IgE elicited by a chemical to a structurally related chemical. For instance, it could be important to a manufacturer or user of pesticides to know if a person hypersensitive to 2,4-D would react to 2,4,5-T or if a person hypersensitive to captafol would react to captan. No research has been performed to date on possible cross-reactivity of pesticide-specific IgE with related pesticides and only limited work has been performed on cross-reactivity of contact hypersensitivity to pesticides.

The ability of 2,4-D formulations to elicit DTH has not been adequately investigated by this project. Additional studies with the amine and ester formulations need to be performed in guinea pigs using the guinea pig maximization test involving epicutaneous and intradermal sensitizations as well as in mice.

In addition, a systematic comparison of the DTH responses in mice and guinea pigs needs to be performed using chemicals with a range of sensitizing potentials. Modes of administration other than abdominal epicutaneous application in mice should be considered.

The conjugates of 2,4-D and MMA used in this study could be used in several additional ways. The conjugates could be used for the development of ELISA procedures for specific IgE which would eliminate the need for radioactive materials necessary in the PRAST assay. The conjugates could also be used to elicit IgG antibodies specific for 2,4-D and malathion which could be used to detect residues in environmental samples. A comparison of the 2,4-D and 2,4,5-T binding capacity of the antibodies generated by Rinder and Fleeker's (1981) conjugate of the diazonium salt of 2,4-dichloro-5-aminophenoxyacetic acid with the antibodies elicited by the conjugate developed in this study would be interesting.

SUMMARY

This investigation was undertaken to study the ability of 2,4-D, malathion, and captafol to produce allergic hypersensitivity in female BALB/c mice. Delayed-type hypersensitivity was studied using epicutaneous applications of the pesticides on the abdomen for sensitization. Following administration of a challenge dose of chemical to one ear, the ear thickness, accumulation of radiolabelled cells in the ear, and histology of the ear were examined. DNFB, a known sensitizer, was tested simultaneously. 2,4-D and MMA were coupled to KLH and injected intraperitoneally to elicit specific IgE antibodies. DNP-KLH was also administered. An attempt to elicit specific IgE antibodies was made using epicutaneous application of 2,4-D, malathion, and DNFB. The PCA test was used to analyze serum samples from treated mice for 2,4-D- and MMA-specific IgE antibodies. PRAST procedures were developed for specific IgE antibodies and the results of PRAST were compared to the results of the PCA test.

2,4-D and malathion failed to produce DTH or specific IgE antibodies following epicutaneous application. DNFB produced very low titers of IgE. The 2,4-D-KLH and MMA-KLH elicited IgE antibodies following a minimum of two immunizations, thus demonstrating the basic immunogenicity of the pesticides. The titers of MMA-specific IgE were slightly higher than those of 2,4-D-specific IgE but less than those elicited by DNP-KLH.

The PRAST procedures for 2,4-D- and MMA-specific IgE proved to be at least as sensitive as and showed a good correlation with the PCA assay. Variability in the results of PRAST performed on serum samples sharing a common PCA titer was noted. Possible sources of variable results between the two tests are discussed.

Captafol and DNFB produced DTH following epicutanous sensitization for two days or over the period of a month. Pretreatment of the mice sensitized with captafol with cyclophosphamide boosted responses measured at 24 hours post-challenge and in mice challenged with DNFB at all intervals examined. At the doses tested, the sensitizing potential of captafol approached that of DNFB.

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