# CANINE REAGINIC ANTIBODY

## CHARACTERIZATION OF THE SPONTANEOUS ANTI-RAGWEED AND INDUCED ANTI-DINITROPHENYL REAGINIC ANTIBODIES OF THE ATOPIC DOG

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## SUMMARY

Anti-2,4-dinitrophenyl reaginic antibody has been induced in the atopic dog and compared with anti-ragweed reaginic antibody of spontaneous canine atopic hypersensitivity. Immunization of an atopic dog with ragweed hypersensitivity with dinitrophenylated ragweed pollen produced a high and sustained level of canine reaginic anti-DNP antibody. The induced and spontaneous forms of canine reaginic antibodies were shown to be identical in heat lability, sulfhydryl sensitivity, molecular size, charge, in their ability to remain fixed at passively-sensitized dermal sites for a prolonged period, and in their capacity to sensitized homologous white blood cells from normal donors for an immunospecific release of histamine. The induced anti-DNP and spontaneous anti-ragweed reaginic antibodies were physically separated and immunochemically differentiated from six other classes of canine immunoglobulins: $\gamma 2a$, $\gamma 2b$, $\gamma 2c$, $\gamma 1$, $\gamma A$ and $\gamma M$, and shown to be members of a distinct class of canine immunoglobulins analogous to human $\gamma E$ (IgE).

A high incidence of spontaneous atopic hypersensitivity has been observed in the progeny of the atopic dog.

## INTRODUCTION

The occurrence of a spontaneous disease process, closely simulating a human disease, in a lower animal affords an opportunity to study the pathogenesis and pathophysiology of the disease under experimental conditions not obtainable in man. Human atopy has been defined as a predisposition to develop certain clinical forms of hypersensitivity subject to hereditary influences (Loveless, 1941), and frequently is expressed as a seasonal hypersensitivity to pollen antigens. Spontaneous hypersensitivity to pollen antigens in the dog was first described by Wittich (1941). Patterson and co-workers (1960, 1965) and Schwartzman & Rockey (1967, 1969) have defined additional features of the canine atopic state:

The atopic dog characteristically experiences a seasonal disease, the manifestations of which parallel the presence of natural allergenic materials (e.g. ragweed pollens) in the

environment. Initially, the period of clinical manifestations is short and discrete (e.g. limited to the ragweed pollen season). Subsequently, in the majority of cases, the duration of involvement lengthens insidiously as the animal becomes hypersensitive to additional allergens. The disease most commonly begins between 1 and 3 years of age, and once affected the animal maintains its atopic state throughout life. The most prominent clinical signs are the results of self-inflicted trauma, particularly to the face, axilla and digits, secondary to pruritus. Although respiratory distress can be induced experimentally (Patterson, Pruzansky & Chang, 1963), it does not appear to be a feature of the natural disease. A breed predilection and a tendency for the disease to occur in families has been observed (Schwartzman and Rockey, 1967). There is a sex predilection which favours females. The atopic dog displays an immediate urticarial dermal reaction when challenged with the appropriate antigen, and its serum contains heat-labile reaginic antibody demonstrable by a passive transfer Prausnitz-Küstner (PK) reaction in a normal homologous recipient. The signs of the disease are invariably controlled by systemic corticosteroids and sometimes by antihistamines. Hyposensitisation with the offending allergen results in a reduction in the severity and duration of the clinical manifestations (Schwartzman and Rockey, 1967).

The spontaneous reaginic antibody of the atopic dog is comparable to the atopic human reaginic (heat labile homocytotropic skin sensitizing) antibody of the $\gamma E$ (IgE) class (Bennich *et al.*, 1968; Ishizaka & Ishizaka, 1970) in all parameters thus far investigated (Rockey & Schwartzman, 1967, 1969). The reaginic antibodies of both species are incapable of inducing a passive cutaneous anaphylactic (PCA) reaction in the guinea-pig, are inactivated by heating at $56°$ C for 2–4 hr and upon exposure to 2-mercaptoethanol, have a rapid electrophoretic mobility and a sedimentation coefficient of approximately 8–9S, and are recovered on Sephadex G-200 gel filtration in an intermediate zone after the recovery of $\gamma M$-macroglobulins and polymeric (9–15 S) $\gamma A$-globulins, but before the elution of 7S $\gamma G$-globulins (Schwartzman and Rockey, 1967; Rockey & Schwartzman, 1969).

The features which distinguish the response of the atopic dog to antigenic stimulation from the response of the normal dog are of special interest. The present study reports the successful induction of anti-2,4-dinitrophenyl (DNP) reaginic antibody in the atopic dog, and further characterizes canine reaginic antibodies. Studies of the progeny from matings between atopic dogs have furnished additional evidence that genetically determined factors are operative in the development of the atopic state.

## MATERIALS AND METHODS

*Atopic canine clinic population.* One of the authors (R.M.S) has had approximately 260 atopic canine patients under study during the last 12 years. In the present investigation, a dog has been classified as atopic only if it has met all of the following criteria: (1) clinical evidence of seasonal hypersensitivity; (2) positive intradermal skin tests to allergenic extracts; and (3) circulating reaginic antibody demonstrable by passive transfer (PK) reactions. A voluntary central core of thirty-five atopic dogs from clinic population has been utilized to provide sera, white blood cells and tissues for study. The atopic canine clinic members also have served as a study group for the evaluation of therapeutic hyposensitization programmes and the medical control of the atopic state (Schwartzman & Rockey, 1967).

*Establishment of a colony of atopic dogs.* A colony of atopic dogs was established for more extensive studies. Two Mongrel dogs (A-Ti-66 and A-Pa-67) displaying multiple

spontaneous hypersensitivities were donated to the investigators by their owners. Two atopic Beagle dogs and one atopic Irish Terrier dog were purchased after examining 237 animals at commercial breeding colonies by direct skin tests and passive transfer (PK) reactions. Permission was obtained from the owner of an atopic Beagle male dog and an atopic Beagle female dog of unrelated lineage to breed the atopic animals and to purchase the progeny. Six Beagle puppies were born in 1966. Two of the puppies died 3 days post-partum. The remaining two male (A-Sw-1 and A-Sw-2) and two female (A-Sw-3 and A-Sw-4) puppies were housed in outdoor quarters in a rural environment to afford them a natural exposure to pollen antigens. The breeding of A-Sw-2 and A-Sw-3 in 1969 resulted in the birth of three puppies that presently are housed in a rural environment.

*Preparation of dinitrophenylated antigens.* Canine serum albumin was isolated by Sephadex G-200 gel filtration (Rockey & Schwartzman, 1967), and further purified by Pevikon block zone electrophoresis (Müller-Eberhard, 1960). Bovine $\gamma$G-globulin (Fraction II, Armour Pharmaceutical Co., Kankakee, Illinois) was used as purchased. Short ragweed (*Ambrosia artemisifolia*) pollen, dandelion (*Taraxacum officinale*) pollen, and mixed grass pollens were obtained from Hollister-Stiers Laboratories (Spokane, Washington). Proteins and crude pollen preparations were dinitrophenylated by the method of Eisen (1964). Dinitrophenylated proteins were precipitated by adjusting the pH of the solution to 2–3 with 1 N hydrochloric acid. The precipitates were centrifuged, washed 2–4 times with 0·25% (v/v) acetic acid, and taken up in 0·2 M NaCl, 10 mM sodium phosphate buffer, pH 8·0. Soluble dinitrophenylated protein preparations were filtered through columns of Sephadex G-25 in the same solvent. The dinitrophenylated crude pollen antigens contained insoluble materials and were dialysed exhaustively against 0·2 M NaCl, 10 mM sodium phosphate buffer, pH 8·0, at 4°C. A molar extinction coefficient, $\varepsilon$, at 360 nm of $1·75 \times 10^4$ for the dinitrophenyl (DNP) group (Eisen, 1964) was used to determine the extent of dinitrophenylation of soluble antigens. The antigen preparations used were: dinitrophenylated canine serum albumin (DNP-CA) containing 27–35 moles of DNP per 70,000 g of protein; dinitrophenylated bovine $\gamma$G-globulin (DNP-B$\gamma$G) containing 45–53 moles of DNP per 150,000 g of protein; dinitrophenylated ragweed pollen (DNP-RWP); dinitrophenylated mixed grass pollens (DNP-GP); and dinitrophenylated dandelion pollen (DNP-DP). The crude pollen preparations contained large amounts of insoluble and yellow materials which precluded an accurate estimation of the extent of dinitrophenylation from the absorbency at 360 nm.

*Induction of canine anti-DNP antibodies.* Four atopic dogs from the clinic population were utilized for a limited immunization programme. A female Dalmatian dog (Sz) with multiple spontaneous hypersensitivities received a single subcutaneous injection of 10 mg of DNP-CA in saline and was bled bi-weekly for 4 months. A female Beagle dog (Ba) with multiple spontaneous hypersensitivities received a single subcutaneous injection of 10 mg of DNP-CA in sodium alginate adjuvant (Algivant®, Colab Labs, Inc., Chicago Heights, Illinois), and a female wire-haired Terrier dog (Al) with multiple spontaneous hypersensitivities received a single subcutaneous injection of 10 mg DNP-B$\gamma$G in sodium alginate adjuvant. Bleedings were performed 2, 4 and 8 weeks post-immunization. An atopic English Bull dog (Pi) with spontaneous ragweed hypersensitivity received a single subcutaneous injection of 10 mg DNP-RWP in sodium alginate and was bled 1, 2 and 6 weeks post-immunization.

Two members of the colony of atopic dogs were employed in a more intensive immunization programme. Atopic colony member A-Ti-66 with spontaneous ragweed hypersensitivity

initially was exposed to DNP-CA by nebulization on 16 bi-weekly occasions, and was bled on alternate weeks. The antigen was administered with a Bird-Mark 7 respirator equipped with micro-aerosol nebulizer (Bird Co., Palm Springs California) attached to the animal by means of a full-face conical mask. Two millilitres of antigen solution (20 mg of DNP-CA/ ml) was administered after the face mask had been fixed in place for a period sufficient so that the animal's respiratory rate had returned to normal. Subsequently, A-Ti-66 received 10 mg of DNP-RWP in sodium alginate adjuvant subcutaneously, and was bled weekly for 6 weeks. A-Ti-66 then received additional subcutaneous injections of 5 mg of DNP-RWP in sodium alginate adjuvant 6, 18 and 33 weeks following the initial immunization with DNP-RWP, and was bled at bi-weekly intervals for 12 months. A second member of the atopic canine colony, A-Pa-67, displaying spontaneous hypersensitivity to mixed grass pollens and dandelion pollen but not to ragweed pollen, was immunized by 16 bi-weekly subcutaneous injections of 10 mg DNP-B$\gamma$G in Freund's complete adjuvant (Difco Labs, Detroit, Michigan) and bled on alternate weeks. Thereafter, A-Pa-67 was exposed on twelve occasions to bi-weekly administrations of 40 mg of DNP-RWP by nebulization and bled on alternate weeks. This programme was followed by five consecutive daily exposures to 40 mg of DNP-RWP by nebulization with bleedings 1, 2, 3 and 4 weeks post-immunization. A-Pa-67 then received 10 mg of DNP-GP in sodium alginate adjuvant subcutaneously and was bled weekly for 4 weeks, received a second subcutaneous injection of 5 mg of DNP-GP in sodium alginate adjuvant and was bled weekly for 10 weeks, and finally received 10 mg of DNP-DP in sodium alginate adjuvant subcutaneously and was bled weekly for 2 months.

Four non-atopic Mongrel dogs were immunized with dinitrophenylated antigens. One dog received 6 monthly subcutaneous or intramuscular injections of 10 mg of DNP-B$\gamma$G in Freund's complete adjuvant and was bled 1–2 weeks after each antigen administration. Three months thereafter, the dog received 10 mg of DNP-RWP in sodium alginate adjuvant subcutaneously and was bled weekly during a 1-month-period. A second non-atopic dog received 10 mg of DNP-B$\gamma$G in Freund's complete adjuvant, dispersed at multiple subcutaneous sites, on a single occasion, and was bled at bi-weekly intervals for 3 months post-immunization. The animal then received a subcutaneous injection of 10 mg of DNP-RWP in sodium alginate adjuvant, was bled weekly for a month, received a second 10 mg of DNP-RWP in sodium alginate adjuvant subcutaneously and was bled weekly for 2 additional months. A third non-atopic animal was exposed on 6 bi-weekly occasions to 40 mg of DNP-B$\gamma$G by nebulization and bled on alternate weeks. A fourth animal was immunized by 5 consecutive daily exposures to 40 mg DNP-B$\gamma$G by nebulization and bled weekly for 1 month post-immunization.

*Other antisera.* Antisera were induced in rabbits, by repeated subcutaneous administration of antigens in Freund's complete adjuvant, against the following proteins: canine serum; canine colostrum; canine $\gamma$G-globulins isolated by DEAE-cellulose column chromatography and Sephadex G-200 gel filtration (Rockey & Schwartzman, 1967); the proteins contained in Sephadex G-200 gel filtration fractions with peak reaginic antibody activity, isolated from atopic canine anti-ragweed and anti-DNP reaginic sera (Rockey & Schwartzman, 1967; 1969); human $\gamma$A myeloma proteins (Dorrington & Rockey, 1968) and pathologic human $\gamma$M macroglobulins (Montgomery, Dorrington & Rockey, 1969). The rabbit antihuman $\gamma$A-globulin sera were rendered specific for $\gamma$A-globulins by absorption with an excess of serum from an individual lacking both $\gamma$A1 and $\gamma$A2 proteins (Dorrington & Rockey, 1968; Rockey *et al.*, 1964). Anti-human $\gamma$M sera were rendered specific for $\gamma$M-

globulins by absorption with isolated human $\gamma$G-globulins and $\gamma$A myeloma proteins.

*Agar electrophoresis.* Immunoelectrophoresis was accomplished by the micro-technique (Heremans, 1960). Preparative agar electrophoresis was carried out on immunoelectrophoresis slides (Ovary, Bloch & Benacerraf, 1964).

*Gel filtration.* Canine serum proteins were fractionated at room temperature by filtration through three in-series $4 \times 60$ cm columns of Sephadex G-200 in sterile 0·2 M sodium chloride, 2 mM EDTA, 10 mM sodium phosphate buffer, pH 8·0 (Rockey & Schwartzman, 1967). The following antibodies and immunoglobulins were utilized as Sephadex G-200 column standard: canine $\gamma$G-globulins ($s^{\circ}_{20,w} = 6\cdot8S$) (Rockey & Schwartzman, 1967); canine $\gamma$G anti-haemocyanin antibody eliciting a PCA reaction in the guinea-pig (Rockey & Schwartzman, 1967); a polymeric canine $\gamma$A myeloma protein (principal component $s^{\circ}_{20,w} = 9\cdot8S$) (Rockey & Schwartzman, 1967; Dorrington & Rockey, 1969); a pathologic human $\gamma$M-macroglobulin ($s^{\circ}_{20,w} = 18\cdot7S$); monomeric ($s^{\circ}_{20,w} = 6\cdot7S$) and polymeric (principal component $s^{\circ}_{20,w} = 9\cdot6S$) human $\gamma$A myeloma proteins (Dorrington & Rockey, 1969); human reaginic antibody (Rockey & Kunkel, 1962); human isohaemagglutinins with intermediate (9–17S) sedimentation rates, isolated by DEAE-cellulose column chromatography (Rockey & Schwartzman, 1967; Rockey & Kunkel, 1962); and a human $\gamma$A cold agglutinin generously supplied by Dr Howard M. Rawnsley.

*Sucrose density gradient ultracentrifugation.* Diluted antiserum, mixtures of antisera, or serum fractions (0·1–0·2 ml) were layered on 4·8 ml linear gradients of from 10 to 25% sucrose in 0·2 M sodium chloride, 10 mM sodium phosphate buffer, pH 8·0, and centrifuged for 8–24 hours in an SW 65 swinging bucket rotor at either 50,000 or 65,000 rev/min in a Beckman Model L2 or L2-65 ultracentrifuge at 4°. The following proteins were used as sedimentation markers: canine albumin (4·5S); canine $\gamma$G-globulins (6·8S); a polymeric canine $\gamma$A myeloma protein (9·8S) (Rockey & Schwartzman, 1969); a polymeric human $\gamma$A1 myeloma protein (9·6S) (Dorrington & Rockey, 1968); and a human $\gamma$M macroglobulin (19S). Antibody was centrifuged together with two or more markers, and the sedimentation coefficient of the antibody was determined as a linear function of the position of peak activity relative to the markers in the density gradient fractions. This procedure was justified by demonstrating that when three or more proteins with different sedimentation coefficients were simultaneously sedimented, a plot of their positions in the density gradient *vs* their sedimentation coefficients (determined by analytical ultracentrifugation) fitted, within experimental error, on a linear curve.

*Analytical ultracentrifugation.* Sedimentation velocity analyses were performed in a Beckman Model E ultracentrifuge equipped with a RTIC temperature control (Rockey & Schwartzman, 1967).

*Passive transfer reactions.* Canine reaginic antibody was assayed by the Prausnitz-Küstner (PK) reaction in normal homologous recipients. Dermal sites were sensitized with 0·1–0·15 ml of serum or serum fraction, and routinely challenged 24–72 hr later by the intradermal injection of antigen (Schwartzman & Rockey, 1967; Rockey & Schwartzman, 1967). The antigens employed routinely were: ragweed pollen extract, dandelion pollen extract (Hollister-Stiers Lab), mixed grass pollen extract (Hollister-Stiers Lab), DNP-B$\gamma$G and DNP-CA.

*Intradermal extinction titration of ragweed pollen antigens.* Ragweed pollen extract was diluted in 0·15 M NaCl, 10 mM sodium phosphate buffer, pH 7·2, so that 1 ml contained from 1 to $10^3$ PNUs/ml, and 0·05 ml of each dilution was injected into dermal sites on the lateral

aspect of the trunk of nine atopic dogs. The endpoint of an antigen titration was taken as the highest dilution which elicited a clearly positive urticarial reaction.

*In vitro release of histamine from homologous white blood cells.* The direct method of Lichtenstein & Osler (1964) and the indirect method of Levy & Osler (1967) were utilized with minor modifications. White blood cells (WBCs) were isolated from hypersensitive atopic and normal canine blood for the direct and indirect procedures, respectively. Canine red blood cells have an unusually slow sedimentation rate, and the methods which employ dextran for isolating WBCs were found to be unsatisfactory. Excellent results however were obtained when the method of Hulliger & Blazkovec (1967) was employed with minor modifications. A 1·5% (w/v) solution of methylcellulose (grade USP, type MC, Vis 15 cps, Dow Chemical Co., Midland, Michigan) in distilled water was prepared and autoclaved for 10 min at 120°. Eighteen millilitres of this solution was mixed with 10 ml of Hypaque-Na-50% (Winthrop Labs, New York, N.Y.), and 3 ml of the mixture were placed in a $20 \times 175$ mm siliconized borosilicate tube. Four to five millilitres of heparinized blood then were layered carefully on top of the clear methylcellulose-Hypaque solution, and the tubes were incubated in a water bath at 37°C. The red blood cells agglutinated and settled into the methyl-cellulose-Hypaque layer within 30–60 min, and the distinct cloudy zone that remained on top of the suspending medium and contained the WBCs and platelets, was aspirated with a siliconized Pasteur pipette. The aspirates were pooled and centrifuged at 110 *g* for 10 min at 4°C. The plasma-platelet supernatant was decanted, and the WBCs were washed with Tris-A solution (25 mM Tris (hydroxymethyl) aminomethane-HCl buffer, pH 7·6, 0·12 M NaCl, 5 mM KCl, containing 3% (w/v) canine serum albumin). The washed cells were resuspended either in 10 ml of Tris-ACM solution (Tris-A solution containing 0·6 mM $CaCl_2$ and 1 mM $MgCl_2$) for the direct procedure, or in 9 ml of the Tris-A solution containing 4 mM EDTA and 100 mg of heparin (Mann Res. Lab., New York, N.Y.) for the indirect procedure. In the direct procedure, aliquots of cells ($1–1·5 \times 10^7$ WBCs in a reaction volume of 4 ml) were incubated with varying dilutions of antigen for 1 hr at 37°C. In the indirect procedure, 1 ml of reaginic anti-ragweed or anti-DNP canine serum or serum fraction was added to the washed, resuspended cells, and the mixture was incubated at 37°C for 90 min. The cells were centrifuged, the supernatant was decanted, and the sensitized cells were washed with the Tris-A solution and resuspended in 4 ml of Tris-ACM solution. The sensitized cells then were incubated with varying dilutions of antigen for 1 hr at 37°C. The antigens employed in both the direct and indirect procedures were: $10^{-2} - 10^{-6}$ µg/ml DNP-BγG and $10^{-1} - 10^{-5}$ µg/ml ragweed pollen antigen E* (King & Norman, 1962). Concentrations of DNP-BγG antigen greater than $10^{-2}$ µg/ml were found to give nonspecific fluorescence and therefore only concentrations less than $10^{-2}$ µg/ml were employed.

The liberated histamine was freed of cells by centrifugation. The supernatant was deproteinized by the addition of 1·5 ml 8% perchloric acid and centrifugation, and the histamine in the supernatant was extracted initially into butanol and then into hydrochloric acid. A fluorescent histamine conjugate was produced by the addition of 0·1% (w/v) o-phthaldialdehyde (Cal Bio-Chemistry Labs., Los Angeles, California) in methanol, and the fluorophore was quantitated with a Turner Model 111 fluorometer equipped with a voltage regulator and a microcuvette adaptor. The primary filter employed (C7-60) had a peak transmission at 360 nm and the secondary filter (K-48) had a peak transmission at 460 nm.

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* Ragweed pollen antigen E was a generous gift obtained from Dr Phillip S. Norman and Dr Burton Zweiman.

Matched $6 \times 75$ mm borosilicate tubes were used as microcuvettes. Authentic histamine (histamine dihydrochloride, J. T. Baker Chem. Co., Phillipsburg, New Jersey) was used to construct standard curves. A range of concentrations of histamine were prepared in 4 ml of Tris-ACM solution and carried through the extraction procedure. The method employed was capable of detecting histamine at a concentration greater than 5 m$\mu$g/ml. All chemicals utilized in the procedure were fluorometric grade (Harleco, Philadelphia, Pennsylvania). All glassware was borosilicate and had been washed in a 1% (w/v) solution of $7 \times$-solution (Linbro Chemical Co., New Haven, Connecticut) and exhaustively rinsed in doubly-distilled water. The total extractable histamine was determined by adding 1·5 ml 8% perchloric acid to an aliquote of WBCs, vortexing the mixture for 5 min, centrifuging, decanting the histamine ladened supernatant and quantitating the extracted histamine as previously described.

The following atopic and non-atopic animals served as sources of cells and antisera for the *in vitro* studies of the immunospecific release of histamine from canine white blood cells (WBCs): 1. Seven atopic canine clinic patients with spontaneous ragweed hypersensitivity that had received hyposensitization therapy over a 3-year-period, furnished WBCs for the direct procedure and antisera for the indirect procedure. WBCs for the direct procedure were obtained 1 month after the ragweed season. Ragweed pollen antigen E was used as antigen in both procedures. 2. The WBCs of sixty-seven non-atopic members of a colony of Beagle dogs, maintained by Dr Donald F. Patterson at the School of Veterinary Medicine, University of Pennsylvania, were examined for their total extractable histamine content. Cells from selected animals were employed in the indirect procedure. 3. Nine atopic dogs with ragweed hypersensitivity, that had not received hyposensitization injections of ragweed antigens served as sources of reaginic antisera for the indirect procedure. 4. An atopic dog (A-Ti-66) with spontaneous anti-ragweed and induced anti-DNP reaginic antibodies was used as a source of materials for both the direct and indirect procedures. Ragweed pollen antigen E and DNP-B $\gamma$G were employed as antigens.

## RESULTS

*Induction of reaginic anti-DNP antibody in the atopic dog.* Repeated exposure of colony atopic member A-Ti-66 to DNP-CA by nebulization failed to elicit an anti-DNP reaginic antibody. A-Ti-66 displayed a prominent seasonal hypersensitivity to ragweed pollen antigens, and the possibility that components of the ragweed pollen might assist in the induction of reaginic antibody was considered. Crude ragweed pollen therefore was dinitrophenylated and administered subcutaneously in sodium alginate adjuvant. In contrast to the initial negative results, within 1 week after a single injection of DNP-RWP, atopic canine A-Ti-66 serum was capable of transferring a strongly positive anti-DNP urticarial (PK) reaction to normal homologous recipient dermal sites. The titre (reciprocal dilution) of homocytotrophic anti-DNP skin-sensitizing activity was determined in one normal recipient to be 640 and in a second normal recipient to be 320. The anti-DNP PK titre of sequentially obtained sera gradually diminished, and activity was absent from serum obtained 6 weeks after the initial DNP-RWP administration (Fig. 1). A second immunization with DNP-RWP again resulted in a high titre of PK activity. The results of subsequent immunizations of A-Ti-66 with DNP-RWP are summarized in Fig. 1.

A second atopic colony member, A-Pa-67, with spontaneous hypersensitivity to mixed

grass pollens and dandelion pollen but not to ragweed pollen, failed to produce anti-DNP or anti-ragweed reaginic antibodies following either parenteral administration of DNP-BγG or exposure to DNP-RWP by nebulization. The possibility that in this instance grass pollens or dandelion pollen would function as ragweed pollen had with A-Ti-66 was considered. However, the parenteral administration of dinitrophenylated crude grass and dandelion pollens also failed to elicit an anti-DNP reaginic antibody.

The immunization of four atopic canine clinic members with dinitrophenylated antigens resulted in the production of anti-DNP reaginic antibody in two instances. Canine clinic member Sz produced anti-DNP reaginic antibody after a single injection of DNP-CA in
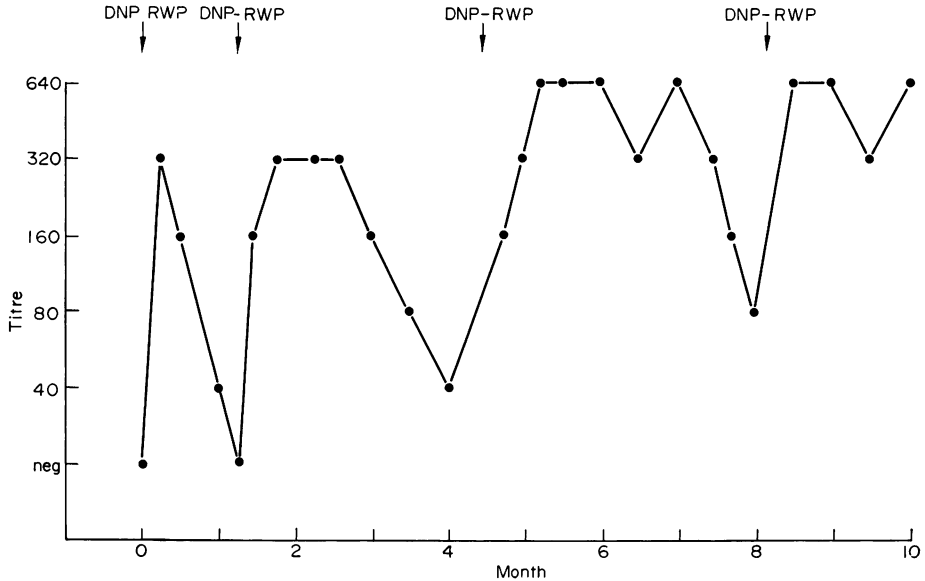


FIG. 1. Induction of reaginic anti-DNP antibody in the atopic dog (A-Ti-66) by immunization with dinitrophenylated ragweed pollen (DNP-RWP). Homologous skin-sensitizing anti-DNP antibody was assayed in two normal canine recipients. Sites were challenged with DNP-BγG 72 hr after sensitization. The titre is the highest reciprocal dilution capable of producing a positive PK reaction.

saline. The antibody was detectable in serum obtained 4 weeks post-immunization but could not be demonstrated in sera obtained thereafter. Atopic canine clinic member Pi produced an anti-DNP reaginic antibody that was demonstrable in serum obtained 1, 2 and 6 weeks after immunization, after a single injection of DNP-RWP in sodium alginate adjuvant. The two remaining atopic clinic members and the four non-atopic dogs immunized with dinitrophenylated antigens all failed to produce reaginic anti-DNP antibody.

*Specificity of canine anti-DNP skin-sensitizing antibody.* The elicitation of a PK reaction in dermal sites passively sensitized with anti-DNP reaginic antibody was not restricted to the immunizing antigen, but could be obtained with dinitrophenylated unrelated proteins. Dermal anaphylactic (PK) reactions were provoked in homologous skin sites, passively sensitized 24–72 hr previously, by an intradermal challenge with either DNP-BγG or DNP-CA, but could not be elicited with bovine γG-globulin (BγG) or canine albumin (CA)

alone. The dermal reactions and the titre of PK activity varied considerably when different normal canine recipients were used, but were reproducible with any single recipient. The urticarial reactions elicited with anti-DNP reaginic sera and dinitrophenylated antigens frequently developed pseudopodia, and in some recipients the acute dermal anaphylactic reactions obtained with low serum dilutions were extremely large.*

*Heat lability of canine anti-DNP PK activity.* Atopic canine sera containing anti-ragweed and anti-DNP homocytotropic skin-sensitizing antibodies were heated in a water bath at 56°C for 2, 3 and 4 hr. Exposure of serum to 56°C for 2 hr resulted in a 50–75% reduction in the anti-DNP PK activity. The 24–72 hr anti-DNP PK activity was destroyed completely by exposing the serum to 56° C for 3–4 hr. The heat inactivation of the anti-ragweed PK activity contained in the same serum paralleled the inactivation of the anti-DNP PK activity.

*Mercaptoethanol inactivation.* Exposure of canine anti-DNP reaginic serum to 0·15 M 2-mercaptoethanol at room temperature for 12 hr, followed by alkylation with 20 mM iodoacetamide by dialysis (Rockey & Schwartzman, 1967; Rockey & Kunkel, 1962), resulted in an 80–95% reduction in anti-DNP PK activity. The inactivation of the anti-DNP PK activity by reduction and alkylation paralleled the inactivation of the anti-ragweed PK activity in the same serum.

*Duration of dermal sensitization.* Atopic canine (A-Ti-66) serum was injected into dermal sites on two non-atopic dogs every other day for 2 weeks. The sites then were challenged with either DNP-BγG or ragweed pollen extract 15 days after the initial sensitization. An immediate urticarial (PK) reaction was observed at all sites, indicating that both the anti-DNP and the anti-ragweed reaginic antibodies remained fixed at dermal sites for at least 2 weeks.

*Electrophoretic mobility of canine reaginic antibodies.* When examined by Pevikon block zone electrophoresis and preparative agar electrophoresis, the anti-DNP and anti-ragweed antibodies of the same serum (A-Ti-66) both had rapid ($\gamma 1$ or $\beta$) electrophoretic mobilities.

*Sephadex G-200 gel filtration.* When atopic canine serum (A-Ti-66) containing both anti-DNP and anti-ragweed homocytotropic skin-sensitizing antibodies was subjected to Sephadex G-200 gel filtration, both activities were recovered in a similar distribution at a volume greater than those at which $\gamma$M-macroglobulins and polymeric (9–17S) $\gamma$A immunoglobulins eluted but before the recovery of 7S $\gamma$G-globulins (Fig. 2a). To demonstrate the resolving capability of the column set, Fig. 2(b) presents the results obtained when a human $\gamma$A cold agglutinin displaying both monomeric (7S) and polymeric (9–17S) cold agglutinin activities (Rockey & Rawnsley, unpublished observation), human $\gamma$A isohaemagglutinins with intermediate sedimentation coefficients (9–17S), and a human reaginic antibody (8–9S) were subjected to gel filtration over the same column set. The cold agglutinin was

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* It has been observed that a normal recipient dog may be utilized repeatedly for PK assay of anti-ragweed reaginic antibody (Schwartzman & Rockey, 1967; Rockey & Schwartzman, 1967). Although the reactivity of any recipient dog is altered (lessened) by repeated testing with ragweed pollen extract, no instance has been observed in which it was not possible to evoke a valid positive reaction in a previously used recipient. In contrast, when testing for anti-DNP reaginic antibody, a normal recipient dog could be utilized only once. The second or subsequent employment of the animal for PK testing with dinitrophenylated antigens not only often gave a negative reaction at the sensitized sites, but also frequently produced a positive wheal reaction at non-sensitized control sites. The false negative reactions at sensitized sites, and the paradoxical reactions at non-sensitized sites were observed as early as 2 days following the initial testing, and for as long as 6 months thereafter. It should be noted that the normal canine recipients may have received parenterally administered dinitrophenol which is utilized routinely as an anthelminthic agent.
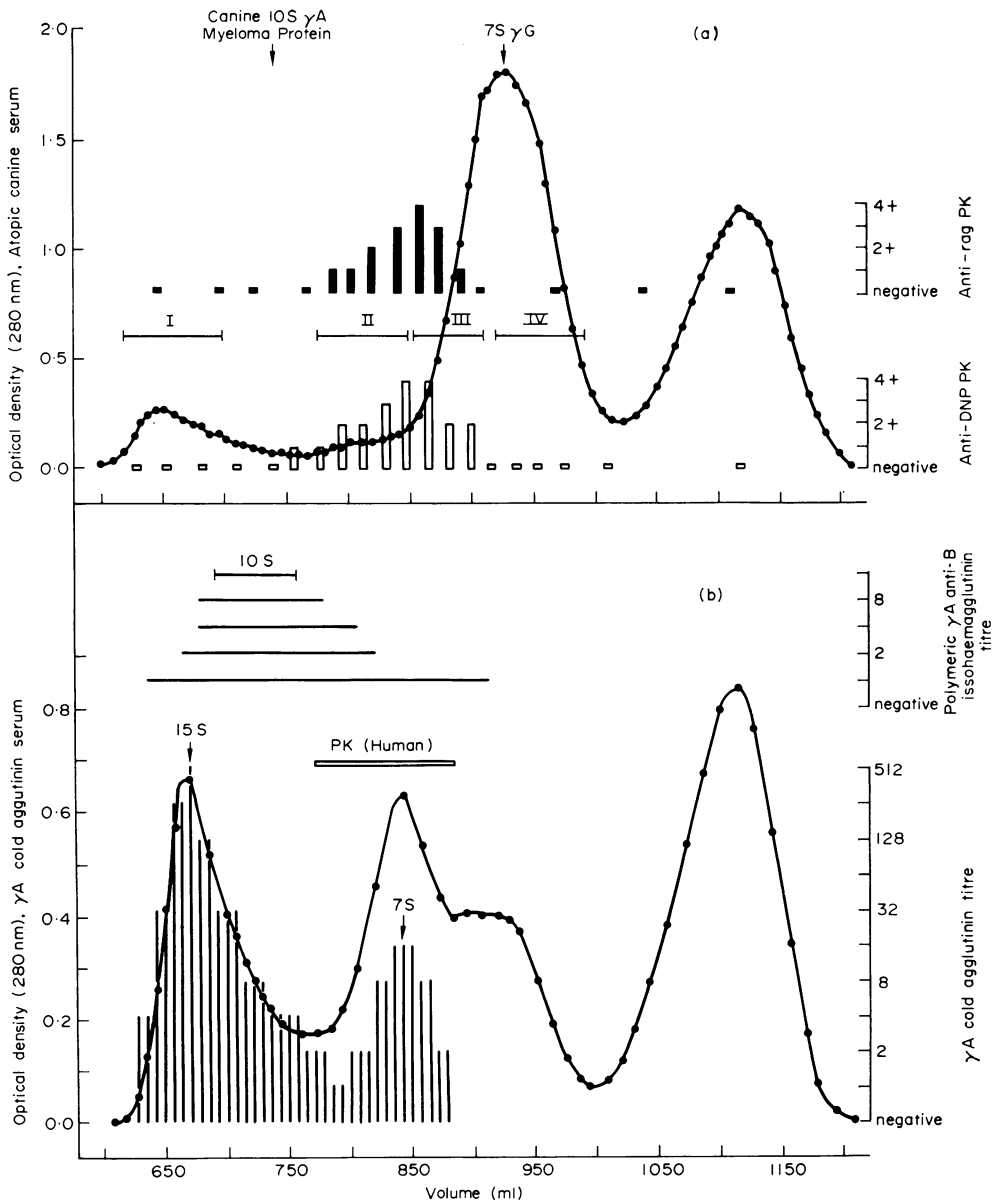
FIG. 2(a). Sephadex G-200 gel filtration of atopic canine (A-Ti-66) serum containing spontaneous anti-ragweed (anti-Rag) and induced anti-dinitrophenyl (anti-DNP) reaginic antibodies. The volumes at which a polymeric (10S) canine $\gamma$A myeloma protein and 7S $\gamma$G-globulins were recovered upon filtration through the same Sephadex G-200 column set also are recorded. Canine $\gamma$M-globulin was identified in fraction I by immunodiffusion studies with an anti-human $\gamma$M serum. (b) The ability of the Sephadex G-200 column set to resolve immunoglobulins with different intermediate (8–17S) rates of sedimentation was defined by filtering a human $\gamma$A cold agglutinin serum, a human reaginic (PK) antiserum, and polymeric $\gamma$A anti-B isohaemagglutinins, isolated by DEAE-cellulose chromatography (Rockey & Kunkel, 1962), over the column set of Fig. 2(a). The sedimentation coefficients for the individual $\gamma$A cold agglutinin fractions and for the pooled anti-B isohaemagglutinin fractions were determined by comparative sucrose density gradient ultracentrifugation (Fig. 3b).

shown to be a γA-globulin by immunochemical analysis of the purified immunoglobulin, isolated by absorption onto red blood cell stroma at 4° C and elution from the washed stroma at 37°C (H. M. Rawnsley, unpublished observation). The anti-A and anti-B isohaemagglutinins with sedimentation coefficients of 9–15S, isolated by DEAE-cellulose column chromatography (Rockey & Kunkel, 1962) and Sephadex G-200 gel filtration, were precipitated by the addition of lyophilized anti-serum specific for γA-globulins, establishing that they also were γA-globulins. Fig. 2(b), in addition, records the sedimentation coefficients of antibodies contained in individual Sephadex G-200 column fractions as determined by comparative sucrose density gradient ultracentrifugation (*vide infra*).
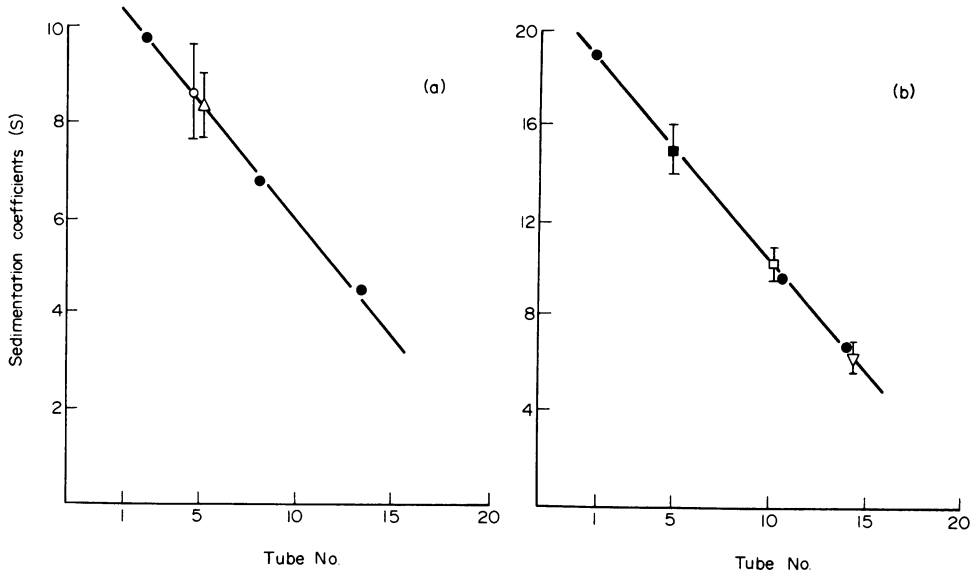


FIG. 3(a). Sedimentation coefficients of canine anti-ragweed (○, anti-rag, PK) and anti-dinitrophenyl (△, anti-DNP, PK) reaginic antibodies determined by comparative sucrose density gradient ultracentrifugation. Canine albumin (4·5S), canine γG-globulins (6·8S) and a polymeric canine γA myeloma protein (9·8S), were used as density gradient sedimentation markers. The position of a component in the density gradient has been plotted as a function of its sedimentation coefficient. (b). Sedimentation coefficients of (■) polymeric and (▽) monomeric γA cold agglutinins and (□) polymeric γA anti-B isohaemagglutinin, isolated by Sephadex G-200 gel filtration (Fig. 2b), determined by comparative sucrose density gradient ultracentrifugation. A human γM macroglobulin (19S), a polymeric human γA myeloma protein (9·6S) and γG-globulins (6·8S) were employed as sedimentation markers.

*Sucrose density gradient ultracentrifugation.* The induced anti-DNP reaginic antibody sedimented on sucrose density gradient ultracentrifugation in the same distribution as the anti-ragweed reaginic antibody of the same serum. Both activities sedimented more rapidly than the serum 7S immunoglobulins, and the PCA (guinea-pig) activity contained in canine γG-globulins ($s°_{20,w} = 6·8S$) isolated from an anti-haemocyanin serum. Clear resolution of the reaginic antibody activity from the 7S immunoglobulin peak required prolonged centrifugation sufficient to sediment the 19S proteins to the bottom of the density gradient tube. The sedimentation coefficients obtained for the canine anti-DNP and anti-ragweed reaginic antibodies were $8·3 \pm 0·4$ and $8·4 \pm 0·65$ respectively (Fig. 3a). Fig. 3b summarizes

the results of density gradient ultracentrifugation analysis of human antibodies with inter-
mediate rates of sedimentation contained in individual Sephadex G-200 gel filtration frac-
tions of Fig. 2(b). The Sephadex G-200 gel filtration column set was capable of resolving
antibodies with different intermediate (8–17S) rates of sedimentation. The human 7S
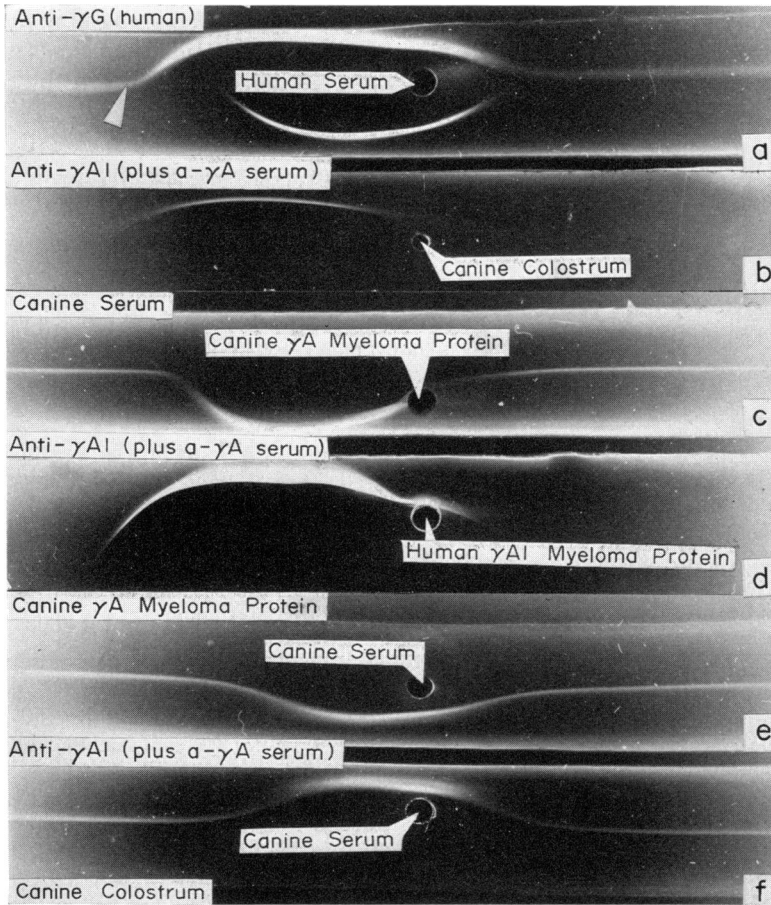


FIG. 4. Immunoelectrophoretic identification of canine $\gamma$A-globulins, utilizing a rabbit anti-
serum prepared against a human $\gamma$A1 myeloma protein and rendered specific by absorption
with the serum of an individual lacking $\gamma$A1 and $\gamma$A2 proteins (a-$\gamma$A serum) (a). In slides c,
e, and f either canine serum, the canine polymeric $\gamma$A myeloma protein or canine colostrum was
diffused from the lateral trough (Wadsworth & Hanson, 1960) to demonstrate the identity of
the $\gamma$A-globulins from the three sources observed with anti-$\gamma$A1 (plus a-$\gamma$A serum). A rabbit
anti-canine colostrum serum also has been identified which reacts with class-specific heavy
chain antigenic determinants of human $\gamma$A-globulins (unpublished observation).

$\gamma$A cold agglutinin and human 7S $\gamma$A myeloma proteins were recovered before the 7S $\gamma$G-
globulins on Sephadex G-200 gel filtration in a distribution similar to that of human and
canine reaginic antibody activities, but sedimented more slowly than the reaginic antibody
activities on sucrose density gradient ultracentrifugation, illustrating that the molecular

parameters responsible for the resolution of distinct immunoglobulins upon density gradient ultracentrifugation and Sephadex gel filtration are not necessarily identical.

*Immunochemical differentiation of canine reaginic antibody from canine γA-globulins.* Rabbit antisera prepared against purified human γA myeloma proteins were rendered specific for γA-globulins by absorption with an excess of serum from an individual lacking both γA1 and γA2 proteins (a— γA serum) (Rockey *et al.*, 1964), as illustrated in Fig. 4. Of six antisera examined, one (F15 anti-γA) was found which specifically precipitated the isolated polymeric canine γA myeloma protein and formed a single precipitin line with the

TABLE 1. Immunospecific precipitation of atopic canine anti-ragweed and anti-DNP reaginic antibodies. Reciprocal dilution PK titres were determined for each absorbed and control serum in a single homologous recipient. The antigens employed were ragweed pollen extract (RWP) and dinitrophenylated bovine γG-globulin (DNP-BγG)

| Atopic canine serum | Antigen | PK titre (reciprocal dilution) |
|---|---|---|
| A-Ti-66 serum | RWP | 4096 |
| | DNP-BγG | 512 |
| A-Ti-66 serum absorbed with anti-γA* | RWP | 2048 |
| | DNP-BγG | 512 |
| A-Ti-66 serum absorbed with anti-γE† | RWP | < 16 |
| | DNP-BγG | < 16 |

* Canine serum absorbed with a rabbit antiserum prepared against a human γA1 myeloma protein and rendered specific for human and canine γA-globulins by absorption with a human serum lacking γA1 and γA2 proteins (Fig. 4).

† Canine serum absorbed with a rabbit antiserum prepared against a reaginic antibody-rich fraction from Sephadex G-200 gel filtration of atopic canine serum. The rabbit antiserum had been absorbed with γM and reaginic antibody-free 7S immunoglobulin Sephadex G-200 gel filtration fractions, and with canine γG-globulins isolated by DEAE-cellulose column chromatography (15 mM sodium phosphate buffer, pH 8·0). The canine γM-globulins of the first Sephadex G-200 gel filtration fraction (Fig. 2, fraction I), used for absorption, were identified with an anti-human γM serum. The γ2a, γ2b, γ2c, and γ1 immunoglobulins of the reaginic antibody-free 7S immunoglobulin Sephadex G-200 fraction (Fig. 2, fraction IV) were identified with antisera specific for the canine immunoglobulin classes (Halliwell, Schwartzman & Rockey, unpublished observations). After absorption, the rabbit antiserum (anti-γE) did not react with either canine γM, γ2a, γ2b, γ2c, or γ1 proteins including a γ2a, a γ1, a γM and two γ2c myeloma proteins which have not been reported previously (R. E. Halliwell, unpublished observations). The characterization of this antiserum and of the canine γE(IgE)-globulin will be reported in more detail elsewhere.

related proteins of γ1 (*β*) electrophoretic mobility present in canine serum and colostrum (Fig. 4). The γA-globulins in an atopic dog (A-Ti-66) serum containing both anti-ragweed and anti-DNP reaginic antibodies, were specifically precipitated with an excess of R15 anti-γA (absorbed with a-γA serum). A ratio of 2 volumes of R15 anti-γA (absorbed) to 1 volume of A-Ti-66 serum resulted in complete precipitation of all of the γA-globulins. The absorbed A-Ti-66 serum supernatant and control A-Ti-66 serum were titrated for residual anti-ragweed and anti-DNP PK activity (Table 1). Immunospecific precipitation of all of the γA-globulins from the atopic canine serum did not diminish the anti-ragweed and anti-DNP reaginic antibody activities, demonstrating that neither were γA-globulins.

*Immunospecific precipitation of canine reaginic antibodies.* A rabbit antiserum, prepared

against the reaginic antibody fraction from Sephadex G-200 gel filtration of atopic canine serum A-Ti-66 (Fig. 2a, pool II), was absorbed with the proteins contained in the $\gamma$M (Fig. 2a, pool I) and reagin-free 7S immunoglobulin (Fig. 2a, pool IV) gel filtration fractions and also with canine $\gamma$G-globulins isolated by DEAE-cellulose column chromatography. The absorbed serum was assayed for its capacity to precipitate the anti-ragweed and anti-DNP reaginic antibodies of serum A-Ti-66 (Table I). Even when the rabbit antiserum had been absorbed with an excess of canine $\gamma$M-globulins, and 7S immunoglobulins of slow and rapid electrophoretic mobility, it retained the capacity to immunospecifically remove both the spontaneous anti-ragweed and induced anti-DNP PK activities, indicating that the canine reaginic antibody activities were associated with a unique class of immunoglobulins analogous to human $\gamma$E (IgE)-globulin.

*In vitro release of histamine from canine white blood cells.* No measurable histamine (i.e. less than 5 m$\mu$g/ml) was released when the white blood cells from seven atopic dogs with spontaneous anti-ragweed hypersensitivity, and one atopic dog (A-Ti-66) with both spontaneous anti-ragweed and induced anti-dinitrophenyl hypersensitivities were employed in the direct procedure, even though circulating reaginic antibodies were demonstrable in their sera. The total extractable histamine of the white blood cells from the eight atopic animals was examined and found to be below the sensitivity of the procedure employed (less than 20 m$\mu$g/$10^7$ WBCs). An effort therefore was made to locate non-atopic dogs with white blood cells containing measurable quantities of extractable histamine, to serve as cell donors for the indirect procedure. White blood cells from fifty non-atopic Beagle dogs were tested to determine their total extractable histamine content. Only three of the fifty Beagle dogs examined furnished cells yielding satisfactory amounts of histamine: P13, 120–160 m$\mu$g/$10^7$ WBCs; P83, 160–240 m$\mu$g $10^7$ WBCs; and P99 120–200 m$\mu$g/$10^7$ WBCs.

P83 and P99 both were the progeny of P13. The fifty non-atopic Beagle dogs were members of a larger colony of animals maintained by Dr Donald F. Patterson for experimental purposes, and the study of the white blood cell histamine content therefore was extended to include the other related members (Fig. 5). A total of six dogs with more than 120 m$\mu$g/g of extractable histamine/$10^7$ WBCs were located within the family group of twenty-one animals.

White blood cells from P13, P83, and P99 were used successfully, together with reaginic anti-ragweed and anti-DNP antibodies from the atopic dogs, in the indirect procedure. These results are summarized in Tables 2 and 3. The extinction titration of ragweed antigen by direct skin testing, the reaginic antibody PK titre, and the extent of histamine release from passively sensitized white blood cells are recorded in Table 2 for nine atopic dogs with ragweed hypersensitivity. Table 3 illustrates the dependence of the immunospecific release of histamine from white blood cells, passively sensitized with either anti-ragweed or anti-DNP reaginic antibodies, upon the concentration of antigen. The maximum release of histamine was obtained over a narrow range of antigen concentration, and histamine release was inhibited by an excess of antigen. The capacity of the anti-ragweed and anti-DNP reaginic antisera to passively sensitize non-atopic canine white blood cells for an immunospecific release of histamine was destroyed by heating the antiserum at 56°C for 4 hr (Table 4).

The atopic canine anti-ragweed and anti-DNP antibodies capable of passively sensitizing homologous white blood cells for an immunospecific release of histamine were recovered on Sephadex G-200 gel filtration in the same distribution as the PK skin-sensitizing activity (Table 4). The pooled fraction containing the canine $\gamma$M-macroglobulins and the fraction

from the second principal protein peak containing the maximum concentrations of canine 7S immunoglobulins of $\gamma$ and $\beta$ electrophoretic mobilities, were devoid of white blood cell-sensitizing activity (Fig. 2a, Table 4). Heating the Sephadex G-200 gel filtration fraction with maximum histamine releasing capacity at 56°C for 4 hr destroyed the activity. The
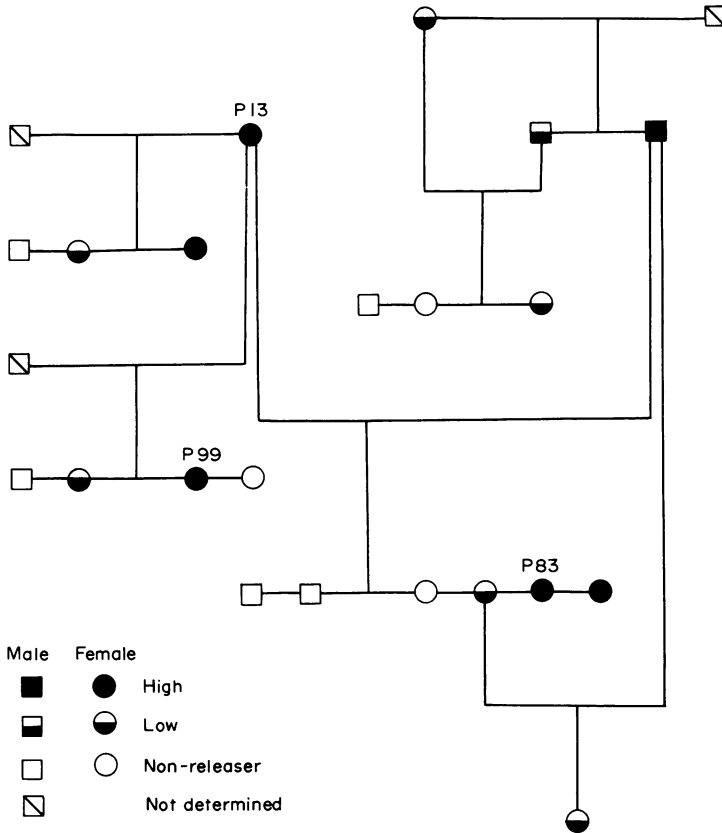


FIG. 5. Familial occurrence of high (greater than 120 m$\mu$g/$10^7$ WBCs), low (40–120 m$\mu$g/$10^7$ WBCs), and non-releaser (less than 40 m$\mu$g/$10^7$ WBCs) levels of total extractable white blood cell histamine in members of an inbred colony of non-atopic Beagle dogs. The results are not consistent with either a simple recessive or simple incomplete dominant hypothesis, if one assumes that the high releasers are homozygous for a gene producing high histamine levels, and the moderate releasers are heterozygous for this same gene. They are consistent with the inheritance of an autosomal dominant gene mutation controlling histamine release if one assumes that the gene only determines whether release occurs, the amount of histamine released depending on other factors (possibly genetic or non-genetic).

same reaginic antibody preparation retained 70–80% of its white blood cell-sensitizing capacity after exposure to 56°C for 30 min (Table 4).

*Familial occurrence of canine atopic hypersensitivity.* A high incidence of spontaneous hypersensitivity to environmental antigens has been observed in the progeny of atopic dogs (Fig. 6). The four members of the canine colony (A-Sw-1, A-Sw-2, A-Sw-3, A-Sw-4) obtained from the mating of two atopic dogs, all developed evidence of atopic hypersensitivity to

*R. M. Schwartzman, J. H. Rockey and R. E. Halliwell*

TABLE 2. A comparison of the intradermal extinction titration of ragweed pollen antigen by direct skin testing, and the PK titre and white blood cell-sensitizing capacity of circulating anti-ragweed antibody for nine atopic dogs with ragweed hypersensitivity. The dogs had not received hyposensitization therapy. Direct skin tests and PK tests were elicited with ragweed pollen extract. Histamine release from passively sensitized white blood cells was induced with ragweed pollen antigen E. The range of concentrations of antigen E employed in the indirect histamine release procedure was $10^{-1}$ to $10^{-5}$ $\mu$g/ml. The maximum percentage of total histamine released is recorded

| Atopic dog | Direct dermal reaction (PNUs/ml) | PK titre (reciprocal dilution) | Histamine release (% of total) |
|---|---|---|---|
| Sz | 16 | 320 | 100 |
| Qu | 125 | 160 | 100 |
| Ze | 125 | 160 | 22 |
| Sc | 125 | 40 | 48 |
| Jo | 32 | 40 | 65 |
| Wh | 63 | 80 | 0 |
| So | 1000 | 80 | 40 |
| Al | 63 | 160 | 50 |
| Pi | 125 | undiluted | 8 |

TABLE 3. Immunospecific release of histamine from non-atopic canine (P83) white blood cells passively sensitized with atopic caninere-aginic anti-ragweed and anti-DNP serum from A-Ti-66

| Tube No. | Antigen ($\mu$g/ml) | Histamine release (m$\mu$g/ml) | % of total released |
|---|---|---|---|
| | Antigen E | | |
| 1 | $10^{-1}$ | 3 | 8 |
| 2 | $10^{-2}$ | 32 | 84 |
| 3 | $10^{-3}$ | 2 | 5 |
| 4 | $10^{-4}$ | 1 | 3 |
| 5 | $10^{-5}$ | 0 | 0 |
| Total release | — | 37 | 100 |
| | DNP-B$\gamma$G | | |
| 1 | $10^{-2}$ | 1 | 3 |
| 2 | $10^{-3}$ | 2 | 5 |
| 3 | $10^{-4}$ | 39 | 100 |
| 4 | $10^{-5}$ | 4 | 10 |
| 5 | $10^{-6}$ | 1 | 3 |
| Total release | — | 39 | 100 |

environmental antigens by 2 years of age. Within 1–2 years after birth, each dog manifested clinical signs of atopy and displayed positive direct intradermal urticarial reactions and circulating reaginic antibody capable of eliciting a positive passive transfer (PK) reaction to one or more of the following allergenic extracts: mixed ragweed, dandelion, grass mix, tree mix, English plantain, sheep sorrel, kochia, marsh elder, goldenrod, lamb's quarter, yellow dock,

alfalfa, house dust, wool, mixed molds, feathers and pigweed. The familial tendency to develop the atopic state also was evident in four additional family units from the clinic population (Fig. 6). In a family of Dalmatians in which the dam and sire both were atopic, all of five progeny developed the atopic state. In a family in which the dam was atopic, one of three progeny developed atopic hypersensitivity. In two families in which the sire was atopic, three of seven and two of three progeny manifested atopic disease. A breed predilection for the development of the atopic state in wire-haired Terriers and Dalmatians

TABLE 4. Immunospecific release of histamine from white blood cells passively sensitized with reaginic antiserum and serum fractions before and after exposure to 56°C. Antigen concentrations employed were $10^{-1}$ to $10^{-5}$ μg/ml for ragweed pollen antigen E and $10^{-2}$ to $10^{-6}$ μg/ml for DNP-BγG. Maximum percent of total histamine release obtained is recorded.

| Serum or serum fraction | Antigen | Histamine release (% of total) |
|---|---|---|
| A-Ti-66 | Antigen E | 87 |
| | DNP-BγG | 100 |
| A-Ti-66 heated (56°C for 4 hr) | Antigen E | 0 |
| | DNP-BγG | 0 |
| Sephadex G-200 fractions* | | |
| I | Antigen E | 1 |
| | DNP-BγG | 0 |
| II | Antigen E | 50 |
| | DNP-BγG | 56 |
| II heated (56°C for 30 min) | Antigen E | 40 |
| | DNP-BγG | 42 |
| II heated (56°C for 4 hr) | Antigen E | 0 |
| | DNP-BγG | 0 |
| III | Antigen E | 22 |
| | DNP-BγG | 25 |
| IV | Antigen E | 0 |
| | DNP-BγG | 0 |

* Sephadex G-200 gel filtration fractions of A-Ti-66 serum (Fig. 2a).

also has been observed. Of thirty-five atopic animals currently under treatment with repository hyposensitization, five are Dalmatians and ten are wire-haired Terriers.

The occurrence of atopic hypersensitivity in the progeny of atopic animals is significantly more frequent than the occurrence of the disease in a general canine population. Only three animals with spontaneous hypersensitivity were detected when 237 dogs were examined at commercial breeding colonies. During a 2-year-period, 14,379 new canine patients have been seen at the University of Pennsylvania School of Veterinary Medicine. Of these, 1,809 animals, including all dogs with overt signs of atopy, were seen in the Dermatology Clinic. During this period, sixty new cases of canine atopy were observed, corresponding to an incidence of 3·3% in the Dermatology Clinic population and 0·4% in the General Clinic population.
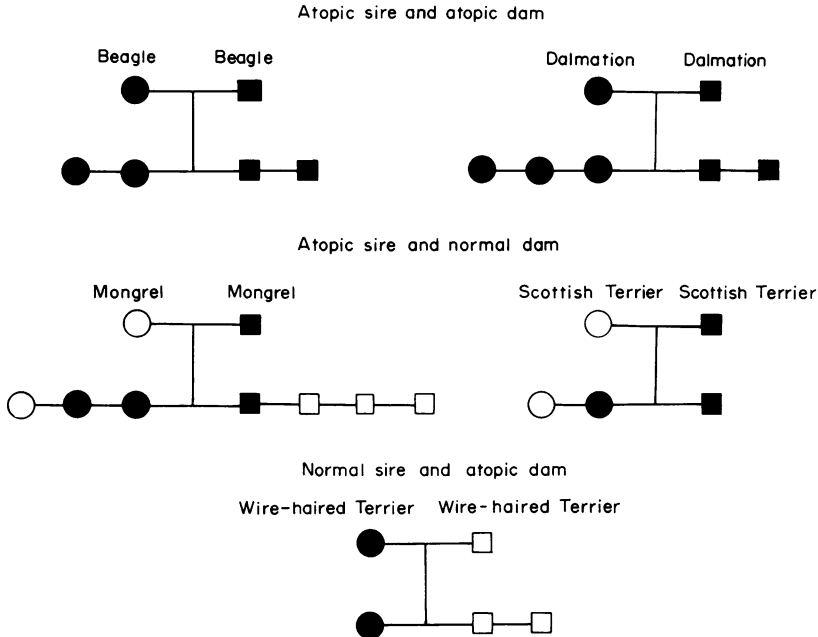
FIG. 6. Development of atopic hypersensitivity in the progeny of atopic dogs after a natural exposure to environmental allergenic materials. ●, Atopic female; ■, atopic male; ○, normal female; □, normal male.

## DISCUSSION

The induced reaginic anti-DNP antibody of the atopic dog paralleled the spontaneously-occurring canine reaginic anti-ragweed antibody, and the reaginic ($\gamma$E, IgE) antibodies of man, in all parameters investigated (Schwartzman & Rockey, 1967; Rockey & Schwartzman, 1967, 1969; Bennich et al., 1968; Ishizaka & Ishizaka, 1970; Rockey & Kunkel, 1962). Each was heat-labile and sensitive to reduction and alkylation, remained fixed at passively-sensitized dermal sites for a prolonged period (more than 2 weeks), had a rapid electro-phoretic mobility, a sedimentation coefficient of approximately 8–9S, and was recovered upon Sephadex G-200 gel filtration in an intermediate zone after recovery of $\gamma$M-globulins and poly-meric (9–17S) $\gamma$A-globulins but before 7S $\gamma$G-globulins. The immunoglobulins of the dog have been shown to be composed of a number of related but not identical classes of proteins (Rockey & Schwartzman, 1967, 1969; Johnson & Vaughn, 1967; Johnson, Vaughn & Swisher, 1967; Fujimoto, 1969; Vaerman & Heremans, 1969). A polymeric canine myeloma protein was identified as a $\gamma$A(IgA)-globulin on the basis of the following criteria: the myeloma protein consisted of a principal component with a sedimentation coefficient ($s^{\circ}_{20, w}$) of 9·8S together with a number of higher polymeric components similar to those typical of human polymeric $\gamma$A myeloma proteins; mild reduction and alkylation reduced the polymers to monomers with a sedimentation coefficient ($s^{\circ}_{20, w}$) of 6·8S; the polymeric canine myeloma protein was recovered following Sephadex G-200 gel filtration in the same distribution as human poly-meric $\gamma$A myeloma proteins, $\gamma$A isohaemagglutinins and $\gamma$A cold agglutinins with similar sedimentation coefficients; the optical rotatory dispersion (ORD) curve of the canine myeloma

protein was similar to those of human $\gamma$A1 and $\gamma$A2 proteins and lacked the Cotton effect at 240 nm typical of $\gamma$G-globulins; an antigenically-related protein was present in higher concentration in colostrum than in serum; the canine myeloma proteins and the related proteins in serum and colostrum were specifically precipitated with an antiserum prepared against a human $\gamma$A1 myeloma protein and rendered specific for $\gamma$A-globulins by absorption with the serum of an individual lacking both $\gamma$A1 and $\gamma$A2 proteins (Rockey & Schwartzman, 1967, 1969; Dorrington & Rockey, 1968). The observation that canine $\gamma$A-globulins may be precipitated with selected antisera prepared against human $\gamma$A-globulins is similar to that reported by Vaerman & Heremans (1969). In addition to $\gamma$A and $\gamma$M proteins, four 7S canine immunoglobulins have been well characterized: $\gamma$2a, $\gamma$2b, $\gamma$2c, and $\gamma$1 (Johnson & Vaughn, 1967; Johnson, Vaughn & Swisher, 1967; Fujimoto, 1969; Vaerman & Heremans, 1969; Vaerman, 1970). The induced anti-DNP and the spontaneous anti-ragweed reaginic antibodies of the atopic dog were separated from polymeric canine $\gamma$M and $\gamma$A proteins by density gradient ultracentrifugation and Sephadex G-200 gel filtration. Precipitation of the $\gamma$A-globulins of atopic canine serum with an antiserum specific for human $\gamma$A-globulins, without loss of PK activity, immunochemically differentiated the canine reaginic antibodies from canine $\gamma$A-globulins. The canine reaginic antibodies also were separated from canine 7S $\gamma$2a, $\gamma$2b, $\gamma$2c and $\gamma$1 protein by density gradient ultracentrifugation and Sephadex G-200 gel filtration. Both the induced anti-DNP and the spontaneous anti-ragweed reaginic antibodies sedimented more rapidly than the 7S proteins. The 7S $\gamma$2a, $\gamma$2b, $\gamma$2c and $\gamma$1 proteins were recovered in the second major peak on Sephadex G-200 gel filtration (Johnson & Vaughn, 1967) after the recovery of the canine reaginic antibodies. In addition, antiserum prepared against the canine reaginic antibodies retained its ability to precipitate the anti-DNP and anti-ragweed reaginic antibodies even after it had been absorbed with an excess of $\gamma$M-globulins and the 7S immunoglobulins contained in the second major Sephadex G-200 gel filtration peak. These findings establish that the canine reaginic antibodies are not $\gamma$M-, $\gamma$A-, $\gamma$2a-, $\gamma$2b-, $\gamma$2c- or $\gamma$1-globulins, but rather are members of a distinct class of immunoglobulins comparable in all parameters studied to human reaginic antibodies of the $\gamma$E (IgE) class. The canine immunoglobulin which displays reaginic antibody activity therefore hereafter will be referred to as canine $\gamma$E (IgE)-globulin.

The Sephadex G-200 gel filtration studies, when combined with the density gradient ultra-centrifugation results, indicate that the canine reaginic ($\gamma$E,IgE) antibodies are closely similar in molecular size to human $\gamma$E (IgE)-globulins. Sephadex G-200 gel filtration in neutral non-dissociating solvents should not be used alone to determine the molecular weight of a protein (e.g. reaginic antibody), as the position of recovery of a protein is not neces- sarily a direct function of its molecular weight. Human 7S $\gamma$A proteins (MW 154,000) and human $\gamma$E(IgE) antibodies (MW 190,000) (Bennich *et al.*, 1968) were recovered at the same volume whereas 7S $\gamma$G-globulins (MW 150,000) eluted after the 7S $\gamma$A-globulins, when filtered through a standard Sephadex G-200 column set (Rockey & Schwartzman, 1967, 1969; Dorrington & Rockey, 1968; Montgomery, Dorrington & Rockey, 1969).

The results of the immunization programme emphasize the fact that the passage of an allergen across a mucosal surface is not a *sine qua non* for the production of reaginic ($\gamma$E,IgE) antibody in the atopic animal. In three atopic dogs, the subcutaneous administra- tion of dinitrophenylated antigens resulted in the production of anti-DNP reaginic antibody. Reaginic antibody production appeared rapidly, occurring within 7 days after antigen administration. The PK activity had decreased or disappeared 6 weeks following the initial

immunization, but as with other molecular forms of antibodies, this decline was followed by a brisk rise subsequent to a second immunization, and a sustained level of anti-DNP reaginic antibody could be maintained by further immunization. Unexpectedly, a reaginic antibody response was not achieved when allergenic material was administered by nebulization. The number of animals and the experimental variations thus far examined are small and more extensive studies will be required to fully define the importance or lack of importance of the route of antigen administration for the production of $\gamma$E (IgE) and other antibodies in the atopic animal. The present experimental results also suggest that the nature of the carrier of the haptenic determinant, or an adjuvant effect of materials contained in the crude ragweed pollen, may be of importance in eliciting a $\gamma$E (IgE) antibody response. In addition, the results suggest that prior spontaneous sensitization of the atopic animal to ragweed pollen antigens may promote a reaginic anti-DNP antibody response to dinitrophenylated ragweed pollen antigens. Here again, more detailed experimental evidence will be required to fully define the roles of carrier and vehicle in eliciting $\gamma$E (IgE) and other antibodies in the atopic animal.

The *in vitro* studies of canine reaginic antibodies, assaying for histamine immunospecifically released from white blood cells, revealed a number of interesting findings. Approximately 95% of the dogs examined had total extractable histamine values less than 20 m$\mu$g/$10^7$ WBCs. Such small amounts do not allow for accurate determinations of histamine release resulting from the interactions of $\gamma$E (IgE) antibody-sensitized WBCs and allergen, employing the present methodology. Thus, the direct procedure utilized in man is not generally applicable to canine studies. The indirect method did prove successful once a normal dog was found whose white blood cell total extractable histamine was in excess of 120 m$\mu$g/$10^7$ WBCs. A familial tendency for the occurrance of high levels of extractable white blood cell histamine was observed in an inbred colony of Beagle dogs. The indirect histamine release procedure and the PK titre are measurements of circulating $\gamma$E (IgE) antibody, whereas the intradermal extinction titration of antigen in the atopic animal is an assessment of fixed reaginic antibody. There is a reasonable correlation between the first two assays in the data presented in Table 2. The factors which determine the ratio of bound to circulating reaginic antibody are obscure and the results of Table 2, comparing the intradermal extinction titrations with the other two assays, emphasize this fact.

The hereditary nature of the atopic state is clearly demonstrated by the results of the breeding experiments. When both the sire and dam were atopic, all of the progeny developed manifestations of atopic disease. When only one parent was atopic, varying percentages of the progeny were affected. The factor or factors responsible for the familial tendency to develop the atopic state again are obscure but are of special interest for further investigations.

The dog is unique among non-primates in that it is the only animal thus far examined in detail, which develops a spontaneous atopic disease closely simulating that of man. The atopic dog therefore offers an especially useful subject for studies on the etiology, pathogenesis, genetics and therapeutic control of atopic hypersensitivity.

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