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Properties of rabbit antiragweed and antihorseradish peroxidase homocytotropic antibodies

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Homocytotropic antibodies (HCA) were produced by the rabbit after primary injection of the dialysis residue of an aqueous extract of dwarf ragweed pollen (DRg) or a purified dwarf ragweed pollen extract (Pool C) only when the antigen was adsorbed on Al(OH)₃. The properties of rabbit ragweed-specific HCA were qualitatively similar to ragweed skin-sensitizing antibodies found in sera of ragweed-allergic human beings, namely: homocytotropic activity (72 hour homologous PCA reaction), heat lability at 56° C., and inactivation by reduction with 2-mercaptoethanol followed by alkylation with iodoacetamide. The molecular weight of rabbit HCA was ~250,000 (Sephadex G-200), somewhat higher than human γ E skin-sensitizing antibodies. Most rabbits immunized with ragweed antigen-Al(OH)₃, systems gave positive conjunctival and intradermal reactions. Intravenous challenge (8 mg. per kilogram) of these animals produced anaphylaxis. HCA were also elicited in response to the immunologic marker, horseradish peroxidase (HRP), when admixed with Al(OH)₃. The properties of HRP-specific HCA (molecular weight ~250,000) were similar to rabbit ragweed-specific HCA. The results suggest that the rabbit can be used as a model to study the biosynthesis of antibodies that have some physico-chemical and biologic characteristics in common with human antibodies involved in immediate hypersensitivity.

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The rabbit has been used frequently as the small experimental animal of choice to study the heterogeneity of ragweed pollen antigen extracts¹⁻⁵ and for a comparison of the properties of antiragweed antibodies, mainly of the γ G blocking (neutralizing) type, with similar antibodies of human origin.⁶⁻⁸ The two species have γ G ragweed antibodies with functional properties that are similar.^{6, 8} Recent reports show that the rabbit can elicit homocytotropic antibodies to dinitrophenyl protein conjugates,⁹⁻¹⁴ bovine serum albumin,^{11, 15, 16} ovalbumin,^{17, 18} tetanus toxoid,¹⁹ *Ascaris suum*,²⁰ *Schistosoma mansoni*,²¹ keyhole limpet hemocyanin, human globulin, bovine globulin, β -lactoglobulin, and thyroglobulin.²² A rabbit dinitrophenyl bovine γ -globulin antibody with homocytotropic activity and physicochemical properties similar to human γ E was described by Ishizaka, Ishizaka, and Hornbrook²³ and tentatively designated as rabbit γ E. In the human, γ E antibodies are now known to be carriers of homocytotropic activity, i.e., skin-sensitizing or reaginic activity.^{24, 25}

It has been shown that the severity of symptoms of ragweed allergy in the human tends to be directly related to serum levels of skin-sensitizing antibody.²⁶ The present investigation was initiated to explore the possibility of using the rabbit as a model to study aspects of the immediate form of hypersensitivity. The objectives were the following: (1) to determine the conditions under which the rabbit might synthesize ragweed-specific homocytotropic antibodies; (2) to compare some physicochemical properties (molecular weight, heat stability, sensitivity to 2-mercaptoethanol) and biologic properties (homologous PCA and active anaphylaxis) of these antibodies with those established for human γ E ragweed skin-sensitizing antibodies; (3) to ascertain if horseradish peroxidase, the immunologic marker,^{27, 28} might also produce homocytotropic antibodies.

MATERIAL AND METHODS

Animals

Randomly bred New Zealand white rabbits weighing between 1.3 and 1.8 Kg. were used for immunization and homologous passive cutaneous anaphylaxis (HPCA) reactions.

Antigens

The dialysis residue (DRg) of a whole aqueous extract of dwarf ragweed pollen and a purified dwarf ragweed antigen (Pool C, 0-60) were prepared as described previously.^{1, 6} Horseradish peroxidase (HRP), with an activity of approximately 260 purpurogallin units per milligram, was obtained from Sigma Chemical Company, St. Louis, Missouri.

Immunization procedure

Six groups of 9 rabbits each were given a primary injection of antigen. The animals of Group I received a 0.2 ml. injection containing 0.2 mg. of Pool C adsorbed on 5 mg. of Al(OH)₃, in each footpad. Group II animals were immunized in a similar manner with the same dosage of DRg adsorbed on Al(OH)₃. Group III animals were immunized in a similar manner with the same dosage of Pool C without Al(OH)₃. Group IV animals received 1.0 mg. of DRg adsorbed on 5 mg. of Al(OH)₃, per footpad. Group V animals received 0.2 mg. of HRP adsorbed on Al(OH)₃, per footpad. Group VI animals were immunized in a similar manner with HRP in the absence of Al(OH)₃. On day 78, the animals were given a second injection identical to the corresponding primary injection. The rabbits were bled from an ear vein on days 5, 9, 13, 21, 28, 42, 78, and 83.

Gel filtration chromatography

Gel filtration was performed through a 2.5 × 90 cm. column of Sephadex G-200 packed with 0.005M phosphate buffer -0.154M NaCl at pH 7.4. A 1 to 2 ml. serum sample was added,

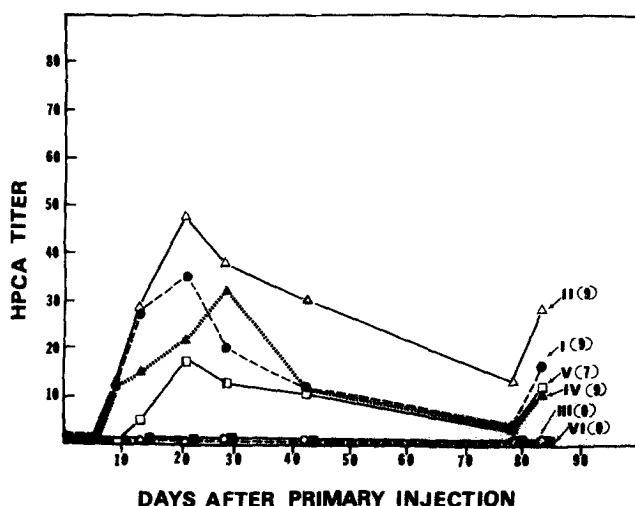


FIG. 1. Homocytotropic immune response in groups of 9 rabbits. The average homologous PCA titers are shown on the ordinate. Group I, Pool C + $\text{Al}(\text{OH})_3$; Group II, DRg + $\text{Al}(\text{OH})_3$; Group III, Pool C, no adjuvant; Group IV, DRg at $5 \times$ the concentration in Group II, + $\text{Al}(\text{OH})_3$; Group V, HRP + $\text{Al}(\text{OH})_3$; Group VI, HRP, no adjuvant. A second injection was given on day 78. Values in parentheses correspond to the number of homocytotropic antibody responders in each group. The peak and range of HPCA titers of groups responding during the primary immune response were: I, 35 (15-80); II, 48 (15-90); IV, 32 (10-50); V, 18 (0-40).

and fractions of 3 to 4 ml. were collected (6 to 8 ml. per hour) by reverse flow. The fractions were assayed spectrophotometrically for protein at 280mM. The molecular weight of homocytotropic antibody was estimated by the gel filtration method of Lindqvist.¹⁹

Homologous passive cutaneous anaphylaxis (HPCA)

Two-tenths milliliter samples of sera or gel filtration fractions, undiluted or appropriately diluted with buffered saline at pH 7.4, were injected intradermally into the backs of shaved rabbits. All antibody samples were assayed in at least 2 rabbits. Normal and heterologous sera as well as buffered saline were used as control systems. After a 72 hour latent period, the animals were challenged intravenously with 1 ml. of 0.9 per cent saline containing 15 mg. of the appropriate antigen and 5 mg. of Evans blue dye. The blue lesions were measured after 45 minutes. The rabbits were killed, skinned, and the blue lesions were then measured on the internal side. A bluing reaction of at least 5×5 mm. was categorized as positive.

Evaluation of heat liability

Homocytotropic antibody samples were heated in a water bath at 56°C . for 2 to 4 hours and then appropriately diluted with saline for the determination of homocytotropic antibody titer by HPCA.

Determination of sensitivity to mercaptoethanol

Serum samples were reduced with mercaptoethanol and alkylated with iodoacetamide as described by Revoltella and Ovary,¹² and then diluted for HPCA.

Conjunctival test, direct skin test, active anaphylaxis

A group of 10 rabbits (Group A) received primary injections of DRg (0.2 mg. per footpad) with $\text{Al}(\text{OH})_3$ as described in the section entitled "Immunization procedure." Six of these animals were injected as before on days 7, 13, and 20. A second group of 8 rabbits (Group B) received a primary injection of DRg (0.2 mg. per footpad) but in the absence of $\text{Al}(\text{OH})_3$. A third group of 6 rabbits were not immunized (Group C). Bleedings were obtained on days 5, 11, 15, 26, and 30. Serum HPCA titers were obtained as described previously.

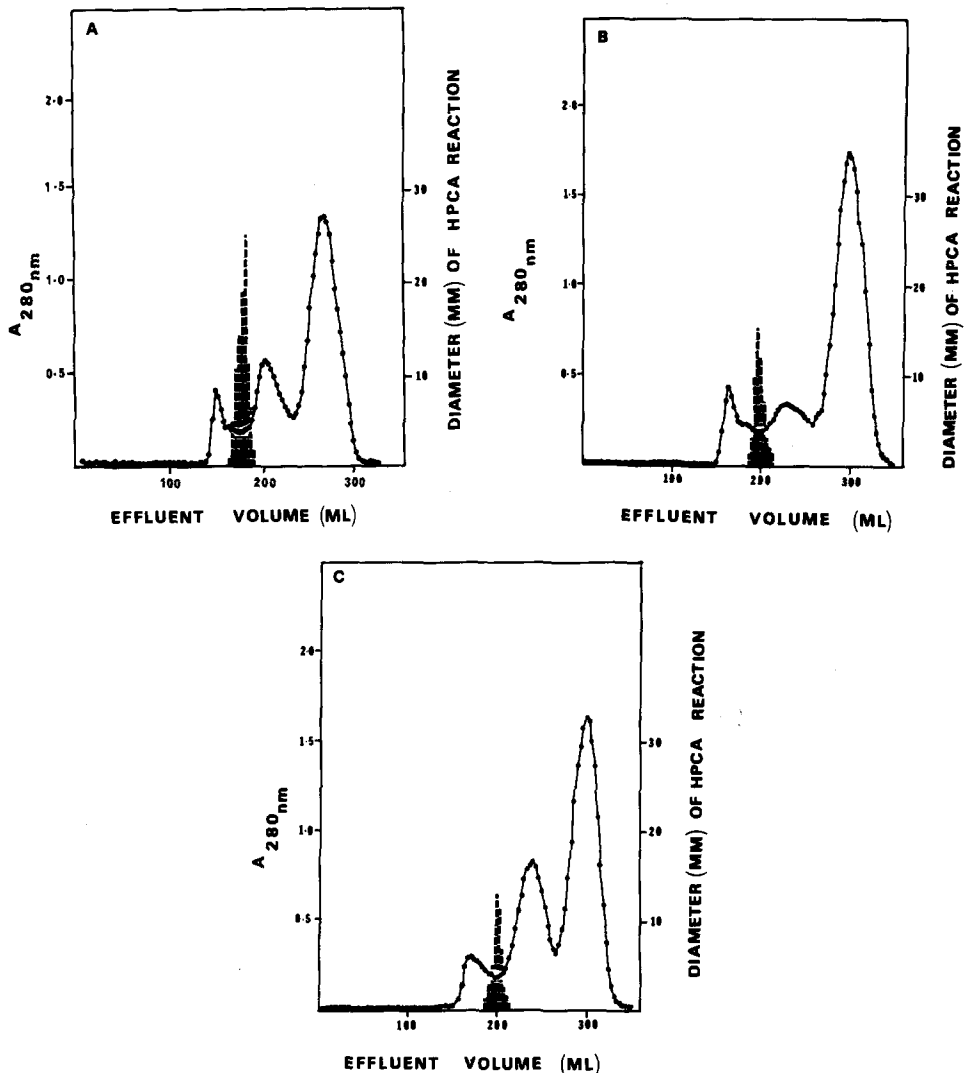


FIG. 2. Gel filtration separation of homocytotropic sera on Sephadex G-200. **A**, Anti-Pool C serum, pooled 15 day bleedings from 3 Group I rabbits. **B**, Anti-DRg serum, pooled 28 day bleedings from 2 Group II rabbits. **C**, Anti-HRP serum, pooled early and late bleedings from 2 Group V rabbits. Magnitude of HPCA reactions given by bars.

Indirect hemagglutination titers were obtained using the tanned red cell method.⁶ PCA titers in the guinea pig were obtained as described previously.⁶ On day 31, each animal was tested for ragweed-specific allergic reactions using conjunctival, intradermal, and active anaphylaxis tests. For conjunctival testing, 0.08 ml. of 0.9 per cent saline containing 0.05 mg. DRg was instilled into the conjunctival sac of one eye (test eye) of the animal, and the same volume of saline was added to the other eye. A positive reaction was recorded if after 15 minutes the degree of erythema, tearing, and edema of the conjunctival membrane and caruncle was greater in the ragweed-challenged eye. At this time 0.5 of DRg in 0.08 ml. saline was instilled into the test eye, and 0.08 ml. saline was again added to the control eye. The eyes were evaluated again after 15 minutes. About 30 minutes before evaluation of active anaphylaxis,

TABLE I. Effect of heating and reduction on 72 hour homologous PCA activity of rabbit homocytotropic antibodies

Serum	Animal group	Day of bleeding	HPCA titer*		
			Untreated	Heated† 10	Reduced and alkylated
Anti-Pool C	I	21	60	10	0
Anti-Pool C (Sephadex fraction of above animal)	I	21	15	5	0
Anti-DRg (Animal No. 1)	II	21	30	5	0
Anti-DRg (Animal No. 1)	II	28	40	10	0
Anti-DRg (Animal No. 1)	II	83	30	5	0
Anti-HRP	V	21	20	5	0
Anti-HRP (Sephadex fraction of above animal)	V	21	10	Undiluted	0
Anti-Pool C (in absence of aluminum hydroxide)‡	III	21	0	0	0
Serum from nonimmunized rabbit§			0	0	0
Heterologous serum (rabbit antidinitrophenyl bovine gamma globulin)			0	0	0
0.9% NaCl			0	0	0

*Reciprocal of highest dilution of serum that produced a positive HPCA reaction.

†Four hours at 56° C.

‡Among 17 animals immunized with either DRg or Pool C but in the absence of Al(OH)₃, none produced HCA.

§Among 9 nonimmunized animals, none gave evidence of circulating ragweed-specific HCA.

each animal was skin tested by injecting intradermally three 0.05 ml. -0.9 per cent saline solutions containing: saline alone, 0.05 mg. DRg, and 0.005 mg. DRg. To facilitate viewing the intradermal reactions, 1 ml. of 0.5 per cent Evans blue in 0.9 per cent saline was given intravenously. The bluing reaction was measured after 15 minutes. Active anaphylaxis was evaluated by challenging each animal intravenously with 1 ml. of 0.9 per cent saline containing 15 to 20 mg. of DRg (approximately 8 mg. per kilogram). Nonimmunized rabbits were used as controls for the conjunctival, intradermal, and active anaphylaxis tests.

RESULTS

The homocytotropic immune response curves corresponding to the average homologous PCA titer for the 6 groups of animals are shown in Fig. 1. The number of responders (producers of HCA) in each group of 9 rabbits is shown in parenthesis. Specific HCA were found in the sera of each animal in which ragweed antigen and Al(OH)₃ was used in the immunization procedure (Groups I, II, and IV). A homocytotropic immune response was not found in any of the animals of the 2 groups in which Al(OH)₃ was omitted (Groups III and VI). HCA were first detected 9 to 13 days after primary immunization and, in most animals, reached peak serum titers between days 21 and 28. At equal dose levels, the purified ragweed sample, Pool C (Group I) and whole extract, DRg (Group II) produced similar immune response curves. DRg, at the higher dose level (Group IV), elicited a somewhat smaller immune response and one that seemed to reach a homocytotropic antibody peak later than DRg at the lower dose level (Group II). The horseradish peroxidase -Al(OH)₃ immune response curve

TABLE II. Active anaphylaxis, conjunctival response, and intradermal reactions in rabbits

Animal No.	HPCA titer before challenge	Indirect hemagglutination titer of heated serum*	PCA titer in guinea pig using heated serum†
Group A Rabbits immunized with DRg in presence of $\text{Al}(\text{OH})_3$			
1‡	80	10	5
2‡	80	5	10
3‡	80	20	5
4	70	5	5
5	70	40	40
6	60	5	5
7	60	5	5
8	50	10	5
9‡	20	5	5
10	10	5	5
	(Ave. 58)	(Ave. 11)	(Ave. 9)
Group B 8 rabbits immunized with DRg in absence of $\text{Al}(\text{OH})_3$			
	0	Ranged between 2 and 20 (Ave. 5)	Ranged between 5 and 20 (Ave. 7)
Group C A group of 6 nonimmunized rabbits			
	0	0	0

*The reciprocal of the highest dilution of antiserum that produced a positive hemagglutination.

†The reciprocal of the highest dilution of antiserum that produced a bluing reaction of at least 1+.

‡Subjective evaluation by two allergists. Positive reaction in test eye indicated more redness than in control animals.

§Measured 15 minutes after intravenous injection of 1 ml. of 0.5 per cent Evans blue.

||Severe: Animal totally immobilized, acute respiratory difficulty, spread-eagle position, head tucked. Moderate: Very lethargic, spread-eagle position, back flattened, ears droopy, moderate anaphylaxis, back curved.

¶Immunized on day 0 only; other animals immunized also on days 7, 13, and 20. The antigen was HRP.

(Group V) was similar in onset, time of peak, and duration of response to that evoked by either of the 2 ragweed-immunized groups in which the dose levels were similar (Groups I and II). On day 78 a second injection was given that was found to evoke a small anamnestic response. Our experience to date, including experiments not reported here, indicates that the rabbit is a good homocytotropic antibody responder to the ragweed antigen- $\text{Al}(\text{OH})_3$ system: 47 of 49 animals were responders. In contrast, no responders were found among 17 animals immunized in the absence of $\text{Al}(\text{OH})_3$. Complete Freund's adjuvant was found far less effective than $\text{Al}(\text{OH})_3$ in the production of a homocytotropic response: one responder was found among 8 animals. The HRP- $\text{Al}(\text{OH})_3$ system elicited a response in 7 of 9 animals. In a group of 9 animals immunized with HRP in the absence of $\text{Al}(\text{OH})_3$, none produced HCA (Group VI).

The results of Sephadex G-200 analysis of 3 homocytotropic sera are shown in Fig. 2. Ragweed-specific (Pool C, Fig. 2, A; DRg, Fig. 2, B) and horseradish peroxidase-specific (Fig. 2, C) homocytotropic activities were found in each case to reside with substances that were of size intermediate of IgM and IgG. The molecular weight of the homocytotropic antibodies was estimated by relating the

immunized with ragweed antigen

Conjunctival test†			Intradermal test‡ (diam. of bluing in mm.)			Degree of active anaphylaxis
Saline	DRg (0.05 mg.)	DRg (0.5 mg.)	Saline	DRg (0.005 mg.)	DRg (0.05 mg.)	
-	+	+	-	13 × 7	15 × 10	Severe
-	-	-	-	10 × 5	13 × 7	Moderate
-	±	+	-	8 × 3	12 × 5	Severe
-	±	+	-	7 × 3	10 × 4	Moderate
-	+	+	-		(Not done)	Severe
-	+	+	-	8 × 3	12 × 9	Moderate
-	+	+	-	9 × 7	15 × 9	Moderate
-	+	+	-	7 × 5	10 × 5	Moderate
-	+	+	-	4 × 3	7 × 5	Mild
-	-	-	-	-	7 × 4	Mild
(All negative)			(All negative)			(No symptoms observed)
(All negative)			(All negative)			(No symptoms observed)

reaction. The sera were preheated for 4 hours at 56° C.
10 mm. in diameter. The sera were preheated for 4 hours at 56° C.
tearing, and edema of conjunctival membrane compared to control eye (0.9 per cent saline) and

to one side and hyperextended, no response to palpitation and noise, urination and massive defeca-
respiratory difficulty, occasional urination and defecation. Mild: Lethargic, not in spread-eagle posi-
correspond to the immune state of animals on the thirty-first day after primary injection of anti-

effluent volume corresponding to peak homologous PCA activity to the effluent
volumes corresponding to peak A_{280mμ} of albumin and IgG.¹⁹ Using molecular
weights of 68,000 and 150,000 for albumin and IgG, respectively, an average
molecular weight value of 250,000 was found for ragweed-specific and HRP-
specific HCA. These values ranged from 240,000 to 270,000.

In Table I are recorded some typical results of heating ragweed-specific sera,
horseradish peroxidase-specific sera, and Sephadex G-200 fractions for 4 hours
at 56° C. In each case, homocytotropic activity was markedly diminished but not
abolished. This was characteristic of sera obtained during the primary as well as
the anamnestic response. Reduction of sera (unheated) with 2-mercaptoethanol
followed by alkylation with iodoacetamide usually resulted in the complete loss
of homocytotropic activity (Table I).

Table II summarizes the results of conjunctival, intradermal, and active
anaphylaxis testing and represents the immune state of animals on the thirty-first
day after primary injection. Positive conjunctival and intradermal reactions
were given only by the 10 animals (Group A) that had been immunized with
ragweed antigen in the presence of Al(OH)₃. The homocytotropic antibody titer

of these animals ranged from 10 to 80. Eight animals that were immunized with DRg in the absence of $\text{Al}(\text{OH})_3$ (Group B) as well as 6 animals that were not immunized (Group C) failed to give positive conjunctival and intradermal reactions. Compared to the intradermal test, the conjunctival test was a less reliable parameter to indicate the presence of HCA. Symptoms of active anaphylaxis were elicited after intravenous challenge of all 8 animals having serum homocytotropic antibodies (Group A). Animals immunized in the absence of $\text{Al}(\text{OH})_3$ (Group B) and nonimmunized animals (Group C) did not exhibit these symptoms. In a typical severe case of anaphylaxis the animals became lethargic, and breathing was irregular several minutes after challenge. Its front and rear legs were straightened out and the animal assumed a spread-eagle position with the head hyperextended to one side. Loss of bowel or bladder control was a frequent symptom. Most animals recovered in 20 to 40 minutes, and no deaths were observed. Although the magnitude of the intradermal reaction did not appear predictive of the degree of anaphylaxis, the severest form of anaphylaxis was produced in animals with the highest HPCA titers.

Further evidence that homocytotropic antibodies are responsible for conjunctival, intradermal, and systemic anaphylactic reactions is found in Table II in the form of indirect hemagglutination and guinea pig PCA data derived from studies using heated (4 hours at 56°C.) sera. The effect of heating the sera of both groups of animals is to decrease the hemagglutination and guinea pig PCA titers by one- to twofold. The sera of the group of rabbits immunized with DRg adsorbed on $\text{Al}(\text{OH})_3$ (Group A) had an average hemagglutination titer and a range of titers only slightly higher than the group of animals immunized in the absence of $\text{Al}(\text{OH})_3$ (Group B). Exclusive of the one animal in Group A (No. 5), the titers of the 2 groups are experimentally similar. In addition, a comparison of the sera of these 2 groups reveals a similarity with regard to PCA titers in the guinea pig. With the possibility that homocytotropic antibody of Group A animals may yet contribute to some extent to hemagglutination and guinea pig PCA titers, these titers are indistinguishable from those of Group B. Therefore, on day 31, conjunctival, intradermal, and anaphylactic reactions appear to be mainly attributable to ragweed HCA rather than another class of immunoglobulins such as IgG.

DISCUSSION

It is apparent from the present study that ragweed-specific HCA, which were induced in the rabbit, have some physicochemical and biologic properties that are similar to ragweed- γE skin-sensitizing (reagin) antibodies of allergic human beings. It was found that rabbit HCA are heat labile at 56°C. , inactivated by reduction with 2-mercaptoethanol, and are involved in conjunctival, intradermal, and anaphylactic reactions. These are well-known characteristics of human γE antibodies.^{29, 32-34} In order to obtain a high frequency of responders to ragweed antigen (either a dialysis residue of an aqueous extract of dwarf ragweed pollen or a purified extract), it was necessary to use $\text{Al}(\text{OH})_3$ in the immunization procedure. The observation that the immunologic marker, horse-radish peroxidase (adsorbed on $\text{Al}(\text{OH})_3$), can evoke HCA suggests that this antigen might find use in studies of the synthesis of HCA.

The molecular weight of both HRP-specific and ragweed-specific HCA was found to be approximately 250,000. This value is somewhat higher than for either the major skin-sensitizing component (M. W. $\sim$ 196,000) of human ragweed-allergic sera²⁹ or an E myeloma protein (M. W. 196,000-200,000).²⁹ Other estimates of the molecular weight of rabbit HCA are 240,000 for antbovine serum albumin¹⁶ and 207,000 for antitetanus toxoid¹⁹ antibodies.

An important group of interrelated observations was that rabbits that had been induced to synthesize ragweed-specific HCA were able to evoke not only conjunctival and intradermal reactions, but also developed anaphylaxis after intravenous challenge. In contrast, animals that were immunized in the absence of Al(OH)₃ did not produce HCA and did not elicit these reactions. Although it was not completely ruled out that other classes of immunoglobulins might participate in such reactions, HCA are strongly implicated at this stage of the immune response (31 days after the first injection of antigen adsorbed on aluminum hydroxide). This view was supported by the finding that heated sera from both groups of animals had low but similar hemagglutination titers and low but similar guinea pig PCA titers.

Although symptomatology in the rabbit is different from that often exhibited by human beings undergoing a constitutional reaction, as in pollen allergy,^{37, 38} the rabbit would appear to merit consideration as a model for immediate hypersensitivity based on a similarity in properties of homocytotropic antibodies in both species.

Nonatopic dogs have been induced to produce ragweed-specific HCA using Freund's adjuvant,³⁰ and recently active anaphylaxis has been demonstrated.³¹ The rabbit would appear to offer certain advantages in these studies, including convenience, economy, homogeneity of strain, and for certain studies in which a short life cycle of the host is advantageous.

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