RABBIT HOMOCYTOTROPIC ANTIBODY

A Unique Rabbit Immunoglobulin Analogous to Human IgE*

By NATHAN J. ZVAIFLER, M.D., and JENNIFER O. ROBINSON

(From the Division of Rheumatic Diseases, Department of Medicine, Georgetown University Medical Center, Washington, D. C. 20007)

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Recently, attention has been directed to a class of antibodies capable of sensitizing the skin of the homologous species for passive cutaneous anaphylaxis. This type of immunoglobulin has been designated as an anaphylactic antibody. The reaginic antibody of man is such an antibody. Whereas it was initially thought that such antibodies were rare in species other than man, it is becoming increasingly apparent that most animals are capable of making skin-sensitizing antibodies, and in some this activity has been identified in several different forms (1).

The rabbit has been shown to make a heat labile homocytotropic antibody, similar in many respects to human reaginic antibody. It appears in the blood at the end of the 1st wk after footpad immunization with dinitrophenyl bovine gamma globulin (DNP-BGG). It disappears from the circulation after a few days or weeks, and does not reappear on subsequent challenge (2). More recent reports have suggested that additional skin-sensitizing antibodies can be identified in rabbit serum. Their time of appearance and characteristics bear a relationship to the type of immunization schedules and antigens employed. Henson and Cochrane have observed that rabbits given footpad injections of bovine serum albumin in complete Freund's adjuvant produce a persistent skin-sensitizing antibody which is heat stable, complement-fixing, and migrates as a slow gamma globulin (3). Richerson et al., examined the production of skin sensitizing antibodies by rabbits immunized with ovalbumin. They found that homocytotropic antibody was best stimulated when the antigen was alum-precipitated and given subcutaneously (4). Low titers of antibody were produced, but unlike the initial report they were sustained for several months. Heating at 56°C for 4 hr decreased the skin-sensitizing activity two- to four-fold, but did not destroy it completely. Lindqvist has shown that footpad injection of alum-precipitated tetanus toxoid results in the formation of a homocytotropic antibody, which is detectable in the rabbit's serum for as long as 133 days after the primary inoculation. An anamnestic response was also observed. He concluded that this antibody was heat stable (5).

This panoply of rabbit skin-sensitizing activities where only recently there were none, raises several questions. Are the various activities limited to the same immunoglobulin class or are they separate immunoglobulins? Do the

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rabbit homocytotropic antibodies belong to any of the previously established immunoglobulin classes? Is sensitivity to heating at 56°C a valid means for separating them? If so, what constitutes a significant difference in heat sensitivity?

In attempting to answer some of these questions, rabbits were immunized with the same antigen, but by different routes, and with different adjuvants. With the different immunization procedures employed, the rabbit homocytotropic antibodies obtained had slightly different physicochemical characteristics, but they all seem to be members of the same class of rabbit immunoglobulins. This class is a new one. It has biologic and physicochemical properties similar to human γE, and also resembles it antigenically. For this reason it has been designated as rabbit γE-globulin. Moreover, it appears hazardous to try to distinguish them on the basis of their heat lability unless careful quantitative analyses are performed. Preliminary reports of this information have been presented elsewhere (6, 7).

Material and Methods

Experimental Animals.—Randomly bred albino rabbits (Arrow Rabbit Farm, Statesville, N. C.) weighing 2000–4000 g were used for immunization and for passive cutaneous anaphylaxis studies. All animals were fed a standard diet.

Antigens.—Bovine globulin (BGG), (Pentex Inc., Kankakee, Ill.); crystalline bovine serum albumin (BSA) (Armour Pharmaceutical Co., Kankakee, Ill.); chicken egg albumin, 5 × crystallized (Pentex). Conjugated antigens were prepared with dinitrophenyl sulfonic acid sodium salt (DNP) (Eastman Organic Chemicals, Rochester, N. Y.), according to the method of Eisen and contained approximately 60 moles of DNP per mole BGG, and 15 moles DNP per mole BSA (8).

Immunizations.—Three routes of immunization were employed—footpad, intraperitoneal, and subcutaneous. Rabbits, usually in groups of six animals, were given injections into all four footpads. The antigen (egg albumin) in phosphate-buffered saline was emulsified with an equal amount of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). The antigen mixture was prepared so as to contain 1.0 mg/ml of protein and approximately 0.25 ml was injected into each footpad. 30 days later a booster of 1.0 mg/ml of egg albumin was given intramuscularly. In the study of immunization by the intraperitoneal route, 31 animals received 2 ml of a mixture containing 5 mg of egg albumin and 0.39 ml of pertussis vaccine containing no less than 25 billion organisms of *Bordetella pertussis*. (Eli Lilly & Co., Indianapolis, Ind.). This mixture was used for both the initial and subsequent injections. These were given at 30-day intervals for 3 months. A group of six rabbits was inoculated subcutaneously with alum precipitated egg albumin. Aluminum hydroxide gel (Amphogel, Wyeth Laboratories, Philadelphia, Pa.) was washed with saline and then resuspended with twice its volume of egg albumin (10 mg/ml) in normal saline. This mixture was allowed to stand for 30 min, centrifuged, and the precipitate obtained was washed with saline. Rabbits were injected subcutaneously with 2 ml of this mixture, which contained approximately 15 mg of egg albumin (EA). Injections were given every other day for 3 doses and repeated twice more at monthly intervals. The immunization schedule was similar to that employed by Richerson (4).

On the 8th to 12th day after each immunization, and at appropriate times thereafter, the animals were bled from the marginal ear vein. The blood collected was allowed to clot at room temperature, separated by centrifugation at 4°C, and then stored frozen, without preservatives at −20°C.

Passive Cutaneous Anaphylaxis (PCA).—PCA[1] reactions in rabbits were performed as described (2). Intradermal injections of 0.1 or 0.2 ml of serum, serum fractions or serum diluted in phosphate-buffered saline (pH 7.4, 0.01 M phosphate) were made into the backs of freshly shaven albino rabbits weighing 2000 or 3000 g. All tests were done in at least 2 animals. After a latent period of 72 hr the animals were challenged intravenously with 1.0 ml of DNP–BSA (20 mg/ml) or 1.0 ml of egg albumin (10 mg/ml) plus 1.0 ml of 5% Pontamine sky blue dye (E. I. duPont de Nemours & Co., Wilmington, Del.) in 0.15 M saline. The resulting lesions were measured and recorded after 30–60 min. The reciprocal of the greatest dilution of serum fraction which gave 5 mm of distinct skin blueing was defined as the PCA titer.

Diethylaminoethyl (DEAE)-cellulose chromatography.—Chromatography was performed with microgranular DE52 cellulose (Whatman, H. Reeve Angel & Co., Inc., Clifton, N. J.). The preswollen cellulose was suspended in starting buffer (0.01 M phosphate, pH 8.0) and adjusted to a pH of 8.0 with 1 N HCl. The cellulose was filtered, resuspended, and the pH rechecked, and it was allowed to stand for 20 min. The "fines" were removed by aspiration. The cellulose was packed under pressure in 2 × 40 cm columns to obtain a flow rate of approximately 180 ml/hour. Serum or serum fractions were dialyzed overnight in the cold against the starting buffer (0.01 M phosphate, pH 8.0). Stepwise elution was carried out at 4°C by the addition of 0.05, 0.10, 0.15, 0.20, and 0.40 M sodium chloride to the 0.01 M phosphate. The protein profile of the eluates was monitored by measuring OD at 280 mμ. The fractions beneath and between the protein peaks were pooled and concentrated. When the protein concentration of a pool was low, then bovine serum albumin was added to give a final concentration of 1 mg/ml. In some experiments a continuous gradient (0.06 M–0.15 M) elution was performed according to the method outlined by Tomasi (9). Conductivity measurements were used to monitor the slope of the gradient.

Gel Filtration.—Chromatography was performed essentially as described (2). A column of 2.5 × 90 cm was packed with Sephadex G-200 which had been equilibrated with phosphate-buffered saline (pH 7.2). Fractions of 4.5 ml were collected by upward flow. Protein profiles were determined by measuring OD at 280 mμ. The columns were calibrated with protein markers of known molecular weights. Human serum albumin (E. R. Squibb & Sons, Inc., New Brunswick, N. J.) and human gamma globulin (Hyland Laboratories, Los Angeles, Calif.), which was refractionated by DEAE-cellulose chromatography, were radiolabeled with ^{131}I and ^{125}I, respectively, according to the method of McConahey (10). Fractions eluted from the column were counted in a 3 in. NaI (thallium-activated) well crystal and the tube containing the greatest number of counts was utilized as the protein peak (Ve). Catalase (Worthington Biochemical Corp., Freehold, N. J.) was extracted into 0.2 M, pH 5.0, citrate buffer according to the method described by Leach (11). When catalase was used as the marker, the column was equilibrated with the citrate buffer; this did not change the elution position of a simultaneously applied albumin sample. The elution position of catalase was designated as the tube which gave the most bubbling after the addition of 10% hydrogen peroxide. The void volume (Vo) of the column was calibrated with Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden). To determine the molecular weight of homocytotropic antibody, 5 ml of the 0.1 M peaks from DEAE-cellulose chromatography were concentrated and then chromatographed. These concentrated fractions contained approximately 3 mg protein/ml and gave PCA titers of 1:20 or greater. They were applied to the Sephadex gel column along with trace amounts of one or more protein markers. Elution was carried out

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[1] Abbreviations used in this paper: BGG, bovine gamma globulin; BSA, crystalline bovine serum albumin; DNP, dinitrophenyl sulfonic acid sodium salt; DNP-BGG, dinitrophenyl bovine gamma globulin; DNP-BSA, dinitrophenyl bovine serum albumin; EA, egg albumin; EDTA, ethylenediaminetetraacetate; NRS, normal rabbit serum; OD, optical density; PCA, passive cutaneous anaphylaxis; P-K, Prausnitz-Küstner.

overnight at 4°C. Samples from each elution fraction were concentrated and used for PCA reactions. The remainder was analyzed for protein and the position of the specific marker.

Protein Concentration.—Fractions from gel filtration and cellulose chromatography were concentrated by positive pressure dialysis with an Amicon Diaflo UM 10 membrane. When the volume of the protein fractions was small, concentration was accomplished by dialysis against a supersaturated sucrose solution by negative pressure. All fractions were dialyzed against phosphate-buffered saline after they were concentrated.

Salt Fractionation.—The zinc sulfate fractionation method of Vaerman was used (12). Equal volumes of serum and $ZnSO_4$ were mixed with stirring; precipitation was induced by adding sufficient 0.1 M Na_2CO_3 solution to bring the pH up to 6.85. After standing for 1 hr the precipitate was removed by filtration through Whatman No. 1 filter paper. The filtrate was kept at a temperature of 28°C for 30 min and the newly formed precipitate was removed by centrifugation. The clear supernatant was freed from zinc by the addition of 1% ethylenediaminetetraacetate (EDTA) salt and dialyzed overnight against phosphate-buffered saline.

Ammonium sulfate precipitation was accomplished by the addition of a volume of ammonium sulfate saturated at 4°C to either an equal volume of serum (50% saturation) or to 2 volumes of serum (33% saturation). The mixture was kept at 4°C for 1 hr. The precipitate which formed was separated by centrifugation, using the high speed head in a PR2 International centrifuge. The supernatant was decanted and the precipitate was dissolved in an amount of phosphate-buffered saline equal to the starting volume of serum. Both fractions were dialyzed against frequent changes of phosphate-buffered saline overnight in the cold.

Heat Stability.—Whole rabbit serum or serum fractions were kept in a constant temperature water bath at 56°C. After a specified period of time, the samples were removed, appropriate dilutions made in phosphate-buffered saline, and they were placed in the shaven skin of rabbits for PCA reactions. The interval between the completion of heating and the intradermal injections did not exceed 3 hr.

Production of Specific Antisera.—

Anti-rabbit gamma globulin (IgG): Rabbit serum was chromatographed on DEAE-cellulose and the fraction eluted with 0.01 M phosphate buffer (pH 8.0) was collected, concentrated, and dialyzed against phosphate saline. The concentrated protein gave only a single precipitin line against a sheep anti whole rabbit serum by immunoelectrophoresis. The IgG was emulsified with complete Freund's adjuvant and was used to immunize guinea pigs following the method of Binaghi (13). Each animal was injected intramuscularly into the hind legs with a total dosage of 1 mg antigen. The same dose was repeated on day 8, again into the hind legs, and intradermally into two sites on the back. Booster injections into the hind legs were made 6 wk later with the same dose of antigen in complete Freund's adjuvant. Blood samples were taken from the retro-orbital plexus. The antisera showed only a single precipitin arc in the cathodal region when tested against normal rabbit serum by immunoelectrophoresis (Fig. 1). The anti-rabbit IgG antisera was absorbed with purified IgA from rabbit colostrum to remove any antibody against light chains.

Anti-rabbit IgA: An anti-rabbit IgA antiserum was prepared with IgA isolated from rabbit colostrum. Colostrum was obtained from albino does by manual milking two days after delivery. Milking was facilitated by a subcutaneous injection of 5 IU of oxytocin (Pitocin, Parke, Davis & Co., Detroit, Mich.). The casein was separated as described by Sell (14) and the clarified supernatant (whey) was chromatographed on Sephadex G-200. The first protein peak from the gel filtration was concentrated and further fractionated by DEAE-cellulose chromatography following the method of Cebra (15). The 0.25 M (sodium chloride) fraction containing the major portion of γA was used to immunize two sheep. Each received an intramuscular injection of approximately 15 mg of antigen emulsified in complete Freund's adjuvant. A booster injection of 10 mg of antigen in complete Freund's adjuvant was repeated 10 wk later. The antisera obtained had antibodies against rabbit immunoglobulins and several

other serum proteins, but after absorption with rabbit IgG and then with fetal rabbit serum (Pelfreez Biologicals Inc., Rogers, Ark.) it was monospecific for IgA by immunoelectrophoresis (Fig. 1).

Anti-rabbit anaphylactic antibody: 30 ml of a serum containing a high titer of skin-sensitizing antibody was treated with zinc sulfate according to the method of Vaerman (12). The super-

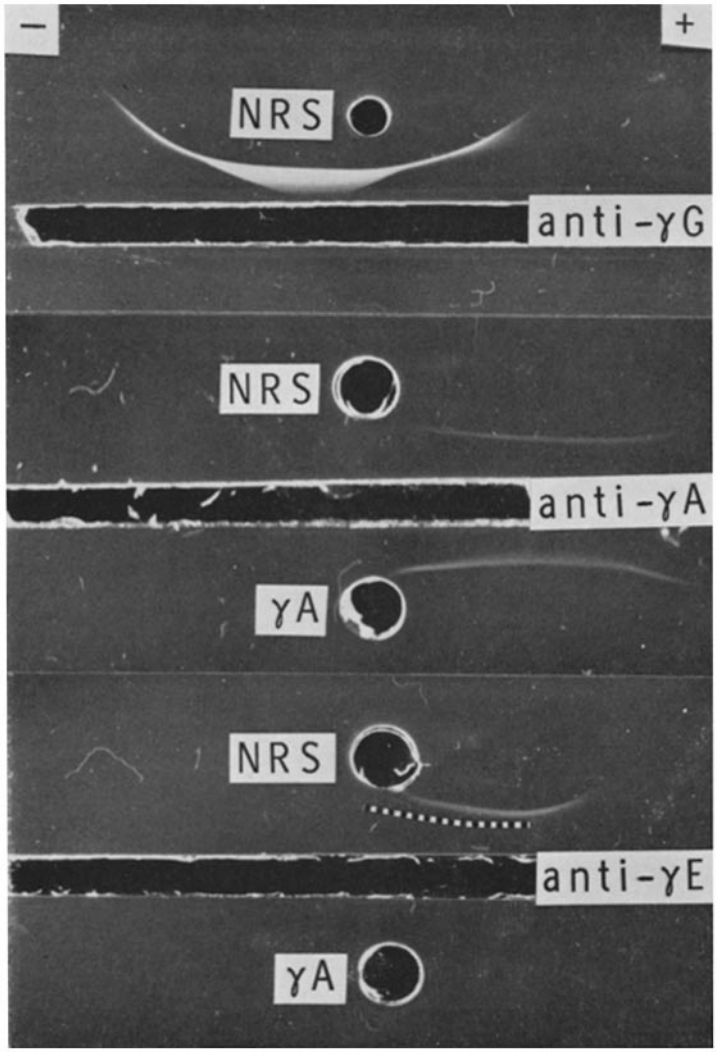

FIG. 1. Immunoelectrophoretic analysis of normal rabbit serum (NRS) or IgA isolated from rabbit colostrum (γA). Guinea pig anti-rabbit IgG (anti-γG), sheep anti-rabbit IgA (anti-γA) and guinea pig anti-rabbit IgE (anti-γE) antiserum were used to develop the patterns. A dense and a fine (designated by the broken line) precipitin arc was demonstrated with the anti-γE antiserum against NRS, but no reactions were obtained with the isolated γA.

natant, which contained the majority of the PCA activity was concentrated and emulsified with complete Freund's adjuvant. It contained 4.8 mg protein and was divided into four equal parts and used to immunize four guinea pigs according to the method of Binaghi (13) outlined above. Booster injections were given at 12 wk using approximately 0.5 mg protein per animal, and at 9 months with 0.75 mg protein per guinea pig. The antiserum obtained (anti-PCA antibody No. 1) consistently removed skin-sensitizing activity from a variety of PCA-positive sera, even after an extensive series of absorptions with fetal rabbit serum, colostrum, and concentrated rabbit saliva (see Results). When it was recognized that there might be more than one kind of rabbit homocytotropic antibody in PCA-positive sera, a second anti-anaphylactic antibody was prepared (anti PCA antibody No. 2). 10 to 20 ml of rabbit sera, which contained high titers of skin sensitizing antibody, was precipitated with 50% saturated ammonium sulfate and chromatographed on DEAE-cellulose. The fractions containing the bulk of the PCA activity (eluted by 0.01 M phosphate, 0.1 M sodium chloride, pH 8.0) were concentrated to 3 mg protein/ml. 5 ml of this material was applied to Sephadex G-200 gel columns. The fractions in the trough between the γM and γG peaks were pooled and concentrated to 0.7 mg protein/ml. This was emulsified with an equal volume of complete Freund's adjuvant and used to immunize two guinea pigs by the method of Binaghi (see above) (13). Each animal was inoculated with approximately 0.5 mg of antigen. A booster injection of 0.5 mg of protein in complete Freund's adjuvant was given to each guinea pig 6 wk later. The antisera produced weak reactions to rabbit gamma globulin and several other serum proteins which migrated anodally when tested by immunoelectrophoresis. Immunoabsorbents were prepared with ethyl chloroformate and used according to the method of Avrameas and Ternynck (16). The antisera were absorbed with insoluble copolymers of rabbit IgG, fetal rabbit serum, and the 0.15 M fraction from DEAE-cellulose chromatography. It produced two precipitin arcs when reacted against rabbit serum in immunoelectrophoresis (Fig. 1), a very fine line beneath the serum well, and another heavier band just anodal to the well. This antisera consistently removed all anaphylactic activity from rabbit serum (see Results below).

Effect of Antihistamine on PCA Reactions.—Rabbits were shaved and injected intradermally with 0.2 ml dilutions of a rabbit serum (anti-EA, intraperitoneal) of known PCA titer. 72 hr later the animals were blued and given 0.1 ml intradermal injections containing 500 μg, 50 μg, and 5 μg of histamine base. After 30 min the area of blueing at each site was measured. The rabbits were given an injection of triprolidine HCl (Burroughs Wellcome & Co., Tuckahoe, N. Y.), 0.5 mg/kg intravenously, followed 5 min later by 5.0 mg of egg albumin. The PCA reactions which developed were measured and then 500, 50, and 5 μg of histamine base was again injected intradermally, and the area of blueing determined. Controls included a group of rabbits who received only histamine, before and after triprolidine, and another which got PCA antiserum, but no histamine.

RESULTS

Patterns of PCA Antibody Response in Rabbits.—It has been shown repeatedly that approximately half of the rabbits receiving footpad immunization with DNP-BGG and complete Freund's adjuvant make a homocytotropic antibody. It appears between the 6th and 8th day postimmunization, remains in the blood for a short period of time, and then is no longer detectable. Repeat immunization does not result in a secondary response. In this study, rabbits immunized with egg albumin into the footpads give somewhat similar results, but the persistence of homocytotropic antibody was noted in a few animals. Approximately half of the rabbits had detectable skin sensitizing activity in their

serum 8 days after immunization (Table I). It was still present on the 16th day. Of the seven positive at this time, four gave PCA reactions which were typical homogeneous deep blue spots, but with three others the skin lesions were more stippled in appearance and less discrete. The antibody was demonstrable with a 1:20 dilution of serum in 3 of 10 animals tested 1 month after the start of immunization. 8 days after reimmunization two of the three had antibody in a titer equal to, or slightly less than, that observed after the first immunization.

Three of six rabbits immunized by the subcutaneous route had detectable PCA antibody in their serum 8 days after the first injection of egg albumin. By the end of 1 month, all but one had PCA activity; some in titers as high as 1:40. When reimmunized there was a small, but definite increase in titer in all

TABLE I

Influence of the Immunization Schedule on the Production of Rabbit Homocytotropic Antibody

Immunization procedure route: adjuvant	Egg albumin injected	No. of rabbits injected	Number of sera containing PCA* antibody on day					
			8‡	16	30	38	68	98
	mg							
Footpad: complete Freund's adjuvant	1	12	6/12§	7/11	3/10	2/10	—	—
Subcutaneous: aluminum hydroxide gel	15	6	3/6	3/6	5/6	6/6	5/5	
Intraperitoneal: *H. pertussis*	5	31	3/11	1/11	1/11	14/27	19/29	16/26

* Passive cutaneous anaphylaxis technique described in Methods section.

‡ Day after immunizing injection. Booster injections given on days 30, 60, and 90.

§ Number of sera containing PCA antibody/number of sera tested.

but one animal. The homocytotropic antibody persisted in the circulation until the second booster dose 1 month later. After the third immunization the titers were again noted to increase (Table I).

Homocytotropic antibody appeared in the circulation of about a quarter of the animals immunized for the first time by the intraperitoneal route. It disappeared in most. After reimmunization, approximately 1 month later, about half of the rabbits had demonstrable PCA activity. It was in higher titer and persisted in all animals who had the antibody. After a third immunization, two-thirds of the rabbits showed skin sensitizing activity; five had PCA titers greater than 1:80 (Table I).

Salt Fractionation of Homocytotropic Antibody.—When whole serum containing homocytotropic antibody was fractionated with 50% saturated ammonium sulfate most, or all, of the PCA activity was recovered in the pre-

cipitate. At 33% saturation, the activity remained in the supernatant. Using zinc sulfate fractionation, approximately half of the activity could be demonstrated in the supernatant fraction. The findings were the same with all sera containing homocytotropic antibody, regardless of the immunization procedure employed.

Heat Stability of Homocytotropic Antibody.—Heating at 56°C decreased PCA activity. The degree to which it diminished depended on the duration of heating

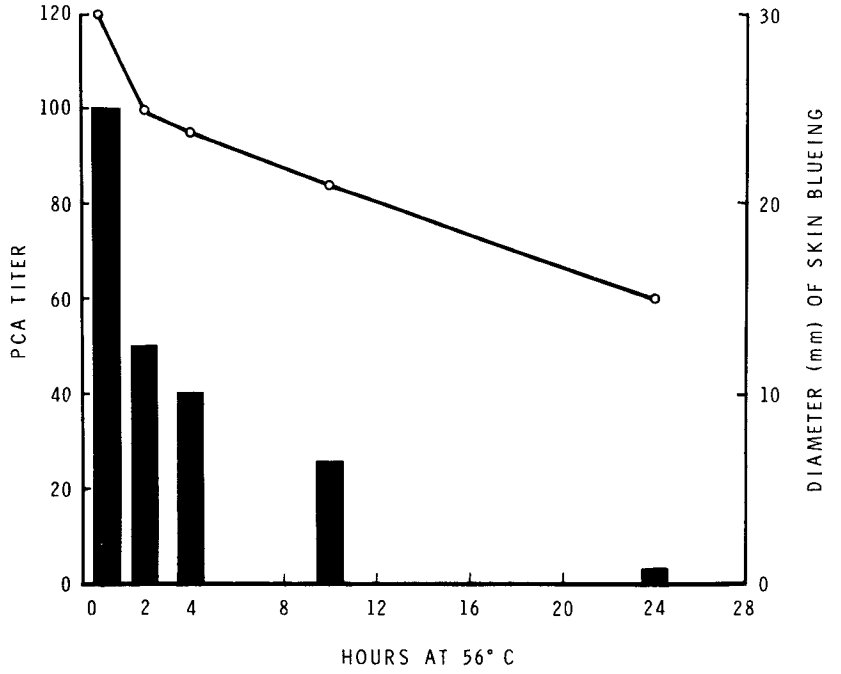

FIG. 2. The effect on rabbit homocytotropic antibody of heating at 56°C for varying periods of time. The PCA titer is shown by the solid bars and the area of skin blueing by the circles (O——O).

and the original titer of anaphylactic activity, but appeared to be independent of the immunization procedure used to elicit the homocytotropic antibody. Heating for 4 hr decreased the original titer approximately 87% (a two- to three-fold fall); continued heating for 24 hr decreased it an additional three-fold. When the starting titer was high, usually 100 or greater, then some residual PCA activity could still be detected at 24 hr. As seen in Fig. 2, as little as 2% of the original anaphylactic activity can still produce a significant degree of skin blueing at 24 hr.

Heat stability studies were performed with sera from 6 rabbits given the

antigen into the footpads, 6 by the subcutaneous route, and 10 intraperitoneally. Homocytotropic antibody from animals inoculated into the footpads or subcutaneously appeared slightly more heat labile than those immunized intraperitoneally. However, the initial titers of PCA activity were three to five times higher in the intraperitoneal group. One rabbit (3-12) of the intraperitoneal group was different from the rest. There was some fall in anaphylactic

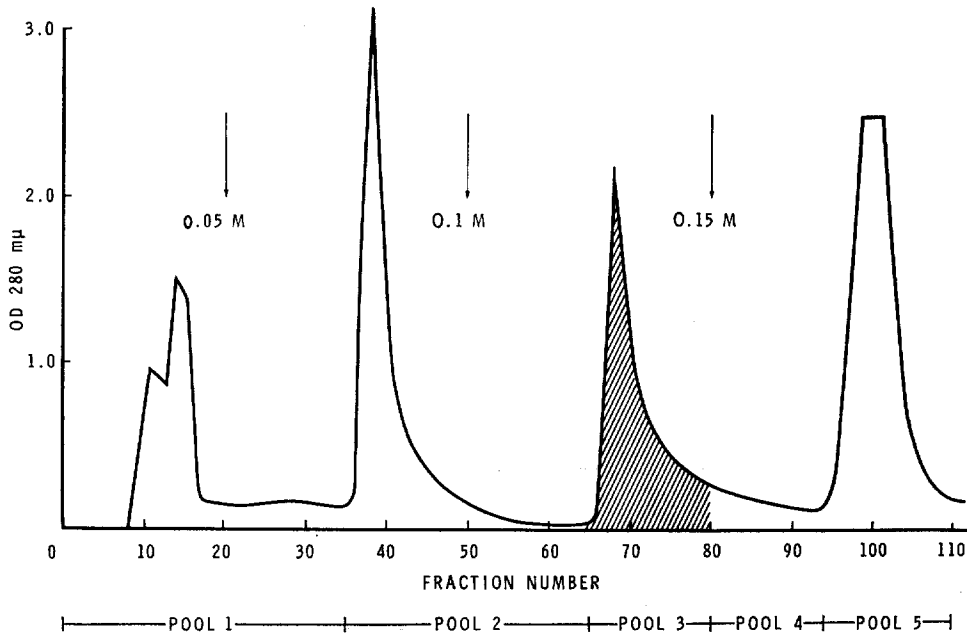

FIG. 3. DEAE-cellulose chromatography of an ammonium sulfate precipitate from a rabbit serum containing homocytotropic antibody (ovalbumin, footpad). The fractions were pooled and tested for skin-sensitizing activity. The shaded area shows the pool which gave passive cutaneous anaphylaxis.

activity after heating at 56°C for 2 and 4 hr, but heating for 24 hr did not produce any significant further decrease. The heat stable homocytotropic antibody in this rabbit appeared only after multiple immunizations. After the first intraperitoneal injection, there was no PCA activity demonstrable. A booster injection 1 month later resulted in an anaphylactic antibody with the conventional heat lability. Booster injections at 60 and 110 days resulted in higher titers of antibody; these displayed the resistance to heating. As will be shown in the next section these same antisera had additional features suggesting the presence of an unusual homocytotropic antibody.

Characterization of Homocytotropic Antibody with Diethylaminoethyl (DEAE)

Cellulose Chromatography.—The distribution of homocytotropic antibody in rabbit serum was analyzed by DEAE-cellulose chromatography. Using stepwise elution, the 0.01 M and 0.05 M peaks contained the bulk of the IgG globulin.

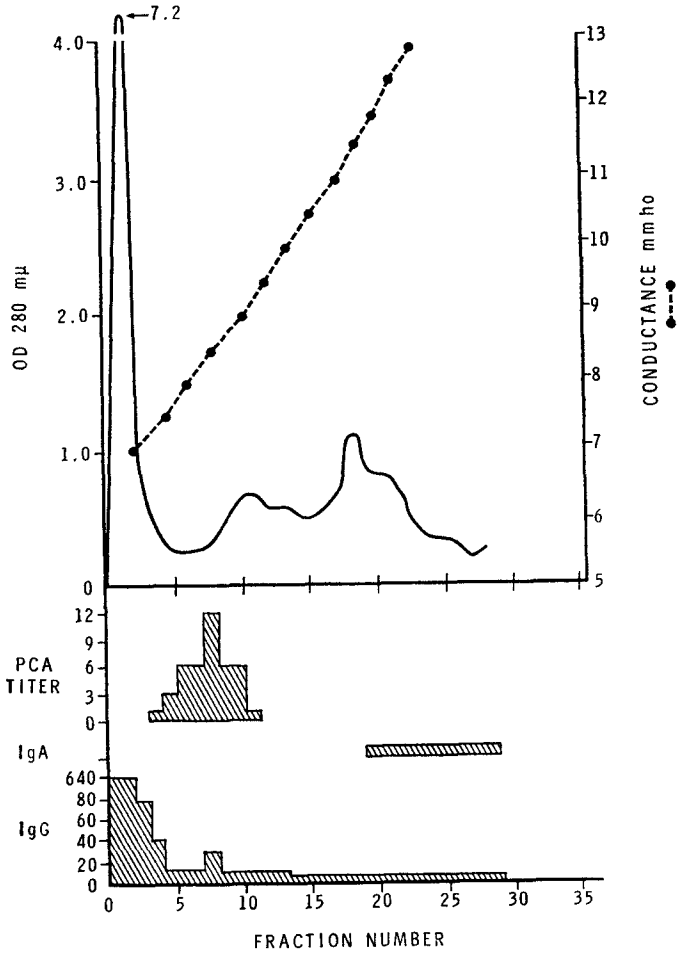

FIG. 4. Continuous gradient elution of rabbit antiserum (DNP, footpad) on DEAE-cellulose. The concentrated fractions were tested for skin sensitizing activity by passive cutaneous anaphylaxis (PCA titer), and the location of the immunoglobulins (IgG and IgA) determined by Ouchterlony double diffusion in agar gel. The greatest dilution giving a reaction is designated as the titer.

The majority of IgA was in the 0.15 M fraction. In all sera examined, the 0.1 M peak contained skin-sensitizing antibody. In 7 studies of the early homocytotropic antibodies produced by footpad immunization (both anti-DNP and anti-EA), the activity was limited exclusively to the 0.1 M fraction (Fig. 3). In

studies with continuous gradient elution the PCA activity appeared in advance of the rabbit IgA (Fig. 4). When antisera from animals which had received multiple immunization (either subcutaneous or intraperitoneal) were chromatographed there was more heterogeneity of skin-sensitizing activity. The 0.1 M fraction still contained the bulk of the activity, but in many antisera it was also detected in the 0.05 M fraction. The PCA titers in both fractions were abolished

TABLE II

Effect of Heating and Absorption with Specific Antiserum on Rabbit Homocytotropic Antibody Eluted by DEAE-Cellulose Chromatography

Serum No.	Immunization procedure route: adjuvant	Elution buffer*	Test performed‡	PCA titer
		M		
128	Intraperitoneal: *H. pertussis*	0.05	control	20
			heat: 4 hr	0
			heat: 24 hr	0
			anti-PCA No. 2	0
		0.1	control	40
			heat: 4 hr	16
			heat: 24 hr	0
			anti-PCA No. 2	0
461	Subcutaneous: aluminum hydroxide gel	0.05	control	6
			heat: 4 hr	0
			anti-PCA No. 2	0
		0.1	control	18
			heat: 4 hr	6
			anti-PCA No. 2	0

* Molarity of saline added to 0.01 M phosphate buffer (pH 8.0).

‡ Control, untreated serum; heat, time at 56°C; anti-PCA No. 2, description of this antibody in Methods section. 1.0 ml of the chromatography fraction was absorbed with 0.1 ml of the antibody at 37°C for 30 min, centrifuged, decanted; repeated twice more. Results were compared to fractions treated similarly with saline.

by heating and by absorption with an anti-PCA antisera (Table II). The 0.05 M fraction appeared to be more heat labile than the 0.1 M fraction. The serum from the rabbit (3-12) which showed the unusual resistance to heating also displayed a distinctive pattern on chromatography. The first appearance of PCA activity was in the 0.1 M fraction, but a significant portion of the skin-sensitizing activity was not eluted until the 0.15 M fraction. This was observed in two separate experiments, and was not seen with any of the other antisera chromatographed.

Molecular Weight Determinations.—The 0.1 M fractions from DEAE-cellulose fractions containing heat labile, skin-sensitizing antibody were applied to Sephadex G-200 gel columns. These columns had been previously calibrated

with radiolabeled human serum albumin, human IgG, and catalase. Each sample studied was chromatographed along with one or more of the markers. The results of four separate experiments with sera obtained by different immunization procedures are shown in Fig. 5. The molecular weights of the three markers are plotted against the effluent volume. Assuming a molecular weight of 68,000

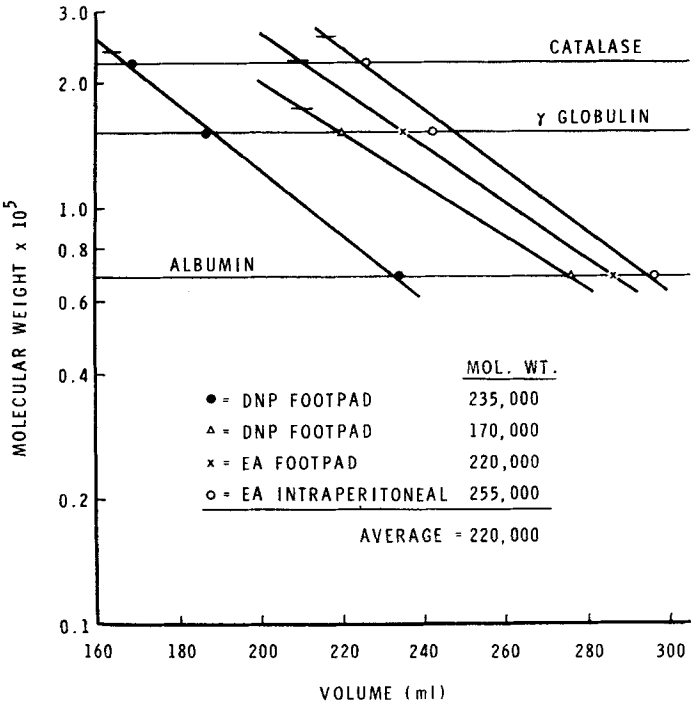

FIG. 5. Determination of the molecular weights of rabbit homocytotropic antibody by gel filtration. The 0.1 M fractions from DEAE-cellulose chromatography were applied to Sephadex G-200 columns. The position of elution of four different samples were compared with markers of known molecular weights—human serum albumin 68,000, human gamma globulin 150,000, and catalase 225,000.

for albumin, 150,000 for IgG and 225,000 for catalase, then the average molecular weight of the homocytotropic antibody from the four experiments is 220,000.

The Effect of Absorption by Specific Antisera on Homocytotropic Activity.—The skin-sensitizing activity of rabbit antiserum was not significantly altered by absorption with a monospecific guinea pig anti-rabbit IgG antisera or with a sheep anti-rabbit IgA antisera. In contrast, absorption with a guinea pig antibody made against the zinc sulfate supernatants of serum from PCA-positive

rabbits (anti-PCA No. 1) completely removed skin sensitizing activity (Table III). In these experiments 0.1 ml of the antisera was added to 1.0 ml of rabbit serum containing skin-sensitizing activity and incubated at 37°C for 30 min. This was repeated twice more. Buffered saline and normal guinea pig serum had no effect on the titers. The anti-PCA No. 1 antisera was absorbed extensively with isolated rabbit IgG, fetal rabbit serum and rabbit colostrum and saliva without affecting its ability to remove PCA activity, but absorption with lyophilized normal rabbit serum eliminated this property.

When the possibility of the heterogeneity of homocytotropic antibody was recognized, a second antibody was produced in guinea pigs using PCA-positive

TABLE III

Effect of Absorption with Specific Antisera on Rabbit Homocytotropic Antibody Activity*

Antiserum				Serum dilution
Anti-IgG	Anti-IgA	Anti-PCA No. 1	Saline	
0‡	0	0	5	1:16
5	5	0	10	
10	10	0	10	1:8
10	10	0	15	
15	12	0	15	1:4
15	15	0	20	
20	15	0	15	1:2
20	20	0	20	

* 1 ml of rabbit serum (EA, footpad) absorbed with 0.1 ml of the antisera at 37°C for 30 min, centrifuged, decanted; repeated twice more.

‡ PCA reactions, diameter (mm) of skin blueing.

fractions from sequential DEAE-cellulose chromatography and gel filtration of rabbit serum. The guinea pig antiserum obtained was absorbed consecutively with insoluble polymers of rabbit IgG, fetal rabbit serum, and then with a 0.15 M fraction eluted from DEAE-cellulose (the fraction shown to contain the bulk of the rabbit IgA). The resultant antiserum, anti-PCA No. 2, showed two precipitin arcs in the region just anodal to the serum well when tested in immunoelectrophoresis against normal or PCA-positive rabbit serum. It did not detect either IgG or IgA immunoglobulins. When the rabbit serum, used as antigen, was heated at 56°C for 4 hr, these lines could no longer be demonstrated (Fig. 6); heating did not have this effect on rabbit IgG or IgA. The heat labile precipitin arcs were present in immunoelectrophoresis with either PCA-positive or negative rabbit serum. Using the anti-PCA No. 2 antibody all homocytotropic antibody activity was completely removed from all PCA-positive serum tested,

regardless of the titer or immunization procedure used to produce them. A serum with a PCA titer of 40 was absorbed with insoluble polymers of guinea pig anti-rabbit IgG. One polymer was made from the anti-IgG antisera absorbed with isolated rabbit colostral γA (anti-H chain specific) and the other from the unabsorbed antisera containing both anti-H and L chains. All PCA activity was removed by the polymer of unabsorbed antisera (anti-H and L chains) but no fall in titer was observed after absorption with the H chain specific anti-rabbit IgG polymer.

Absorption of PCA Positive Serum with an Insoluble Polymer of Ovalbumin.—An insoluble copolymer made with 50 mg of egg albumin and 450 mg of bovine

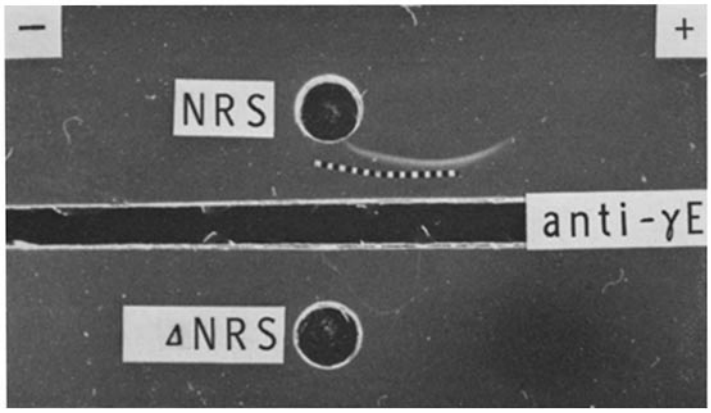

FIG. 6. Immunoelectrophoretic analysis of a normal rabbit serum (NRS) before and after heating at 56°C for 4 hr (Δ NRS). The pattern was developed with the guinea pig anti-PCA No. 2 antiserum (anti γE). A dense and a fine (shown by the broken line) precipitin arc was present before heating. Neither was detected after heating.

gamma globulin (BGG) was used to absorb 10 ml of a PCA-positive antiserum (EA, intraperitoneal). The mixture was gently stirred at room temperature for 2 hr. The flocculant copolymer was separated by centrifugation and the supernatant was removed to test for skin-sensitizing activity. The polymer was washed repeatedly (until the OD_{280} of the last wash was 0.01) and then treated with 0.2 M glycine-HCl buffer (pH 2.2). The eluted protein was neutralized, dialyzed against phosphate-buffered saline, concentrated to approximately 4 ml, and used for PCA testing. Skin-sensitizing activity of the serum was reduced from a titer of 20 to less than 10, and the eluted protein gave a PCA titer of 10. Similar treatment of the same PCA positive serum with an insoluble polymer of BGG alone caused only a slight reduction in the original PCA titer and no activity was demonstrable in the eluate.

Absorption of PCA Positive Serum with an Anti-Human IgE Antiserum.—1 ml

portions of rabbit anti-ovalbumin serum (EA, subcutaneous) were incubated for 90 min at 37°C with 0.6 ml of either a sheep anti-human IgE antiserum[2], a sheep anti-rabbit IgA antiserum or saline. They were then maintained over-night at 4°C. The mixture was centrifuged, the supernatants were decanted and diluted with phosphate-buffered saline and tested for skin-sensitizing activity. As shown in Table IV the anti-human IgE antisera reduced the PCA titer from 20 to 10. The skin blueing was fainter, the lesions were smaller, and they de-

TABLE IV

Effect of Absorption with Anti-Human IgE Antibody on Rabbit PCA Activity*

Antiserum‡			Serum Dilution
Anti-human IgE	Anti-rabbit IgA	Saline control	
0	0	0	1:40
0	0	0	
0	<5§	5	1:20
0	7	11	
0	10	13	1:15
0	11	17	
5	15	17	1:10
10	15	16	
10	20	<20	1:5
11	20	20	

* 1 ml of rabbit serum (EA, subcutaneous) was absorbed with 0.6 ml of saline or antiserum for 90 min at 37°C and over-night at 4°C.

‡ Anti-human IgE, sheep anti-FcND (1518); anti-rabbit IgA, sheep anti-rabbit IgA.

§ PCA reactions, diameter (mm) of skin blueing.

veloped more slowly than the saline control or serum absorbed with the sheep anti-rabbit IgA.

Influence of Triprolidine on PCA Titers.—Rabbits given intradermal injections of 500, 50, and 5 μg of histamine base developed 21, 14, and 10 mm diameter of skin blueing, respectively, (average of 10 experiments). Treatment with triprolidine (0.5 mg/kg) blocked the skin blueing normally produced by these quantities of histamine, but as seen in Fig. 7, it only diminished the PCA titer approximately 75% (two-fold decrease).

Lack of Homocytotropic Antibody in Secretions.—Homocytotropic antibody could not be demonstrated in secretions from rabbits whose sera contained high

[2] The specific sheep anti-FcND (1518) was kindly supplied by Dr. H. Bennich and Dr. S. G. Johansson.

titers of skin sensitizing activity. The secretions were collected after stimulation of flow by 0.3 ml of neostigmine hydrobromide. The major component was saliva with some admixture of nasal and lacrimal secretions. Skin-sensitizing activity was not demonstrated with undiluted samples, or when they were concentrated to 15 mg of protein/ml, even from animals whose sera contained PCA antibody at a 1 :100 dilution.

Conjunctival challenge with egg albumin was performed in six rabbits. Three had no demonstrable homocytotropic antibody, three had PCA activity with

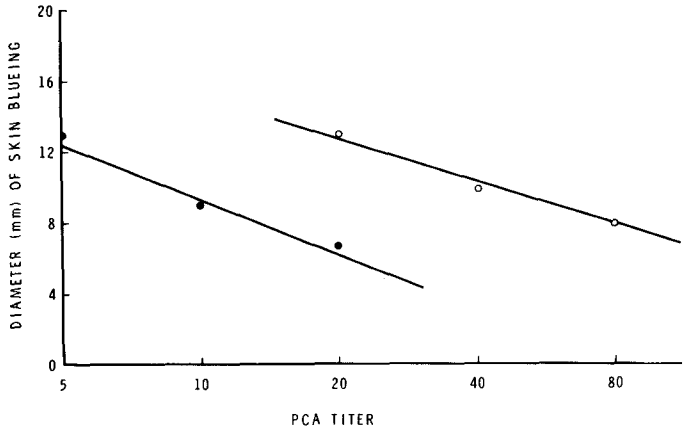

FIG. 7. The influence of triprolidine on rabbit passive cutaneous anaphylaxis. The area of skin blueing produced by varying dilutions of serum containing rabbit homocytotropic antibody was compared in normal controls (○——○) and in rabbits treated with 0.5 mg/kg triprolidine hydrochloride (●——●).

titers of 6, 80, and 200. Two drops of a solution containing egg albumin (1 mg/ml) was placed in one eye and similar amounts of bovine gamma globulin (1 mg/ml) in the other eye. No conjunctival inflammation was observed in either eye in any of the rabbits.

DISCUSSION

The originally described skin-sensitizing substance in the serum of rabbits immunized with the hapten antigen, DNP, was heat labile and migrated in electrophoresis as a fast gamma or beta globulin. It eluted from Sephadex G-200 gel between the IgM and IgG peaks (2). In the present study, some additional features have been observed. Namely, the anaphylactic activity is consistently eluted from DEAE-cellulose with a 0.10 M phosphate saline buffer, and the ability to sensitize rabbit skin is completely abolished by absorption with a specific antiserum. Similar properties have been found for the skin-sensitizing

material present in hyperimmune serum obtained from rabbits given ovalbumin by a variety of immunization schedules. The only difference noted was that the anaphylactic activity from hyperimmune sera appeared in two fractions from DEAE-cellulose chromatography; a portion eluting with the 0.05 M peak, the remainder in the 0.01 M peak. Both fractions, however, were heat labile and inactivated by an anti-PCA antibody.

These physicochemical and immunologic properties provide a better definition of the anaphylactic substance, but several important questions remain. First, what is the evidence that the factor responsible for rabbit passive cutaneous anaphylaxis is an antibody, particularly an antibody of a unique immunoglobulin class? Second, if it is a unique immunoglobulin, are its properties sufficiently like those of human IgE to justify calling the class to which it belongs, rabbit IgE?

Its antibody nature is supported by the following observations: (*a*) PCA reactions are mediated by antibodies in other species (1). (*b*) Onoue and associates demonstrated that an isolated rabbit anti-haptenic antibody (*p*-azobenzenearsonate) could sensitize homologous skin (17). (*c*) The ability to sensitize rabbit skin can be removed by absorption with an anti light chain antisera (5). (*d*) Protein capable of sensitizing rabbit skin was eluted from insoluble polymers of ovalbumin used to absorb PCA-positive hyperimmune (EA) rabbit serum. These results are also in accord with the preliminary report of Ishizaka and associates that anaphylactic antibody can be demonstrated in PCA-positive rabbit serum by radioimmunoelectrophoresis (18).

The contention that the rabbit homocytotropic antibody is an immunoglobulin which is distinct from the other recognized rabbit immunoglobulins is supported by a number of observations. The DEAE-cellulose fractionation procedures employed in the present study and by Lindqvist clearly separated the skin-sensitizing activity from the bulk of the IgA. Anaphylactic activity was not demonstrated in secretions shown to be rich in IgA. In addition, absorption with specific anti-IgA antisera did not effect skin-sensitizing activity, confirming Lindqvist's findings (5). The DEAE-cellulose fractions containing PCA activity also showed a considerable amount of IgG. Likewise, IgG was present in the ammonium sulfate fractions which had most of the protein with skin-sensitizing properties. However, the supernatant from zinc sulfate fractionation had very little IgG, but contained the bulk of PCA activity. The Sephadex gel filtration data showed a clear separation of the homocytotropic antibody from the IgG and IgM immunoglobulins; its molecular weight of 220,000 is larger than the former and smaller than the latter. Moreover, absorption with a specific anti-IgG antisera did not remove skin-sensitizing activity.

The strongest argument that rabbit homocytotropic antibody is a distinct immunoglobulin, analogous to human IgE, comes from the finding that PCA activity could be abolished from rabbit serum completely, and consistently, by

an antisera which gave no reactions against any of the known rabbit immunoglobulins. The ability of this antisera to remove PCA activity was not influenced by extensive absorptions with fetal rabbit serum, rabbit IgG, secretions such as colostrum or saliva, or fractions containing IgA. Significantly, it was blocked by absorption with normal rabbit serum. This antisera (anti-PCA No. 2) did not detect IgG or IgA in rabbit serum by immunoelectrophoresis, but gave distinctive precipitin arcs in the region just anodal to the serum well. This does not necessarily imply that the arcs observed are detecting homocytotropic antibody. Proof for this awaits completion of radioimmunoelectrophoresis studies. It is of interest, though, that both precipitin lines were not demonstrated after the serum was heated at 56°C for 4 hr. Ishizaka has shown a similar heat lability for the human IgE detected by radioimmunoelectrophoresis (19). The demonstration that a portion of the rabbit PCA activity can be removed by absorption with an antibody to human IgE implies that they share some common antigenic determinants.

As noted in the introduction, several groups of investigators have identified one or more rabbit homocytotropic antibodies (2–5). In general, these have been contrasted with the originally described heat labile, rabbit anaphylactic antibody (2). A variety of factors have been shown to influence their production, but the route of immunization and type of adjuvants seems to be important. Henson and Cochrane showed that about half of the rabbits immunized into the footpads with BSA and complete Freund's adjuvant made an early appearing, heat labile homocytotropic antibody. It was not observed with the other immunization procedures they employed. Of great interest was their finding that most of the rabbits also produced a skin-sensitizing antibody whose activity was inhibited by the factor in cobra venom which depletes the test rabbit of C'3. This other skin-sensitizing antibody was also observed in antisera stimulated by the injection of antigen intravenously or into the footpads with complete Freund's adjuvant. The term "complement-dependent PCA antibody" was used to designate this particular homocytotropic antibody. Additional distinguishing features included: its persistence in the circulation for long periods of time, its presence in hyperimmune sera, and physicochemical characteristics suggesting a rabbit IgG antibody. The ability of this homocytotropic antibody to produce vascular permeability was blocked by platelet, neutrophil, or complement depletion, and by an antihistamine, but it was not destroyed by heating at 56°C for 2 hr (3). Lindqvist also described a rabbit homocytotropic antibody which is heat stable and persists in the circulation for long periods of time. It developed after a primary immunization with alum-precipitated tetanus toxoid and increased in titer after booster injections of the antigen (5).

The resistance to heating at 56°C would appear to constitute an important difference between these two homocytotropic antibodies and the one described in this paper. In both of these studies, however, the effects of heating were

determined by using reduction in the number of millimeters of skin blueing obtained with undiluted antiserum as the sole criterion for quantifying PCA activity. As shown in Fig. 2 this can be misleading when high titered antisera are investigated. Under these circumstances large decreases in PCA titer may be associated with only small reductions in the size of the blue spot given by the undiluted serum. As little as 2% of the initial PCA activity gives a significant area of blueing, even after 24 hr of heat inactivation. The slope of the curve in Fig. 2 is very similar to the figure in Lindqvist's paper which is the basis for the claim that the homocytotropic antibody he studied could withstand heating at 56°C for 32 hr. Extrapolation of the data on the effects of heating on PCA activity presented in the paper by Henson and Cochrane shows that increasing the amounts of antibody above 50 μg precipitable nitrogen would result in very little increase in the size of the area of blueing. A similar observation has been made of the P-K (Prausnitz-Küstner) reaction. Wheal areas are roughly proportional to the challenge dose, up to 120 mm; above this size the response curve flattens out (20). These findings suggest that titration of PCA activity affords a more reliable means for assessing the heat lability, or stability, of a homocytotropic antibody. The two- to four-fold decrease in PCA titer noted after heating at 56°C for 4 hr, corresponds to the findings with the homocytotropic antibody studied by Richerson (4). We would like to suggest that the term "heat stable homocytotropic antibody" be reserved for those antisera which show little or no further decrease in PCA titer after 4 hr of heating at 56°C.

The physicochemical properties of the rabbit homocytotropic antibodies described by Richerson, Lindqvist, and in this report are remarkably similar. In each, the skin-sensitizing activity migrated as a fast gamma or beta globulin, and eluted from DEAE-cellulose in a fraction which appears after the bulk of the IgG and before the IgA. The PCA activity is associated with the proteins eluted from Sephadex G-200 in the region between the IgM and IgG peaks. The molecular weight of the rabbit anaphylactic antibody investigated by Lindqvist was calculated to be 207,000 (5), which is very similar to the 220,000 obtained in this study.

The findings of Henson and Cochrane that increased vascular permeability can be produced in rabbit skin by two distinct mechanisms suggests a heterogeneity of rabbit homocytotropic antibodies. This is not too surprising when one considers evidence obtained in other species. Anaphylactic activity appears to reside in two separate immunoglobulin classes in rodents, (21, 22) and passive sensitization of human and monkey tissues may be mediated by immunoglobulins other than IgE (23, 24). We have been unable to demonstrate more than one homocytotropic antibody with a 72 hr latent period in any sera studied, with the possible exception of rabbit 3-12. This rabbit's serum showed remarkable stability when heated at 56°C. There was an initial fall in PCA titer, but little

additional decrease after the 4th hr. Only a portion of the skin-sensitizing activity was eluted in DEAE chromatography with the 0.10 M fraction; the remainder was found with the protein eluted with the 0.15 M phosphate saline buffer. This late appearance of activity was confirmed in several experiments and persisted when only the 0.15 M fraction was rechromatographed. Limited absorption studies were performed with the initial anti-anaphylactic antibody (anti-PCA No. 1). These showed only a partial decrease in PCA titer of the whole serum. Unfortunately, these studies were completed before the more specific anti-PCA No. 2 antibody was available, and the ability of this antisera to remove skin-sensitizing activity from the 0.15 M fraction could not be checked.

The IgG, complement dependent PCA antibody, (3) was characterized in a biologic system (cobra venom treated rabbits) which was not used in the present studies. Therefore no direct comparison can be made. The stippled type of skin blueing produced by the antisera from some animals immunized into the footpads with complete Freund's adjuvant may be indicative of the complement-dependent antibody. This type of skin blueing was not noted with antisera produced by other immunization procedures. However, no PCA activity was found associated with slow ($\gamma 2$) IgG fractions, and the antisera obtained by footpad immunization were inactivated by heating and absorption with anti-PCA antisera.

While human reaginic antibody and the rabbit homocytotropic antibody have many physicochemical characteristics in common, certain differences in their biologic activities should be noted. Reaginic antibody appears in secretions (25–27). We have not been able to demonstrate skin-sensitizing activity in saliva, nasal, or lacrimal secretions; neither has it been found in colostrum (5). There is no ready explanation for this discrepancy, except that systemic immunizations were employed in the rabbits and most human allergies are associated with ingested or inhaled allergens. It may be that the reagins present in external secretions are produced locally. Reaginic antibody can be detected in allergic individuals by conjunctival testing, but in rabbits, whose serum had demonstrable skin-sensitizing antibody, no conjunctival reactions were obtained after local administration of antigen. We have also been unable to produce wheal and flare reactions in the skin of sensitized rabbits. These differences may be due in part to the fact that these hyperimmunized rabbits also have large amounts of IgG antibodies, which may be acting as blocking antibodies.

The finding that an antihistamine drug cannot abolish the PCA reaction in rabbits was similar to the observation of Lindqvist (5), but at variance with Henson and Cochrane (3). The latter authors used very large amounts of Chlorpheniramine (approximately 50 mg per animal) and were able to completely block the skin sensitization produced by both the homocytotropic and comple-

ment dependent PCA antibodies. It is possible that these larger doses are blocking mediators other than histamine. Becker and his associates have shown that triprolidine and mepyramine inhibit the blueing produced in rabbit skin by bradykinin (28). There is no information about the effects of antihistamines on passive anaphylaxis in man, but orally administered antihistamines merely decrease wheal and flare reactions in allergic individuals without abolishing them (29).

SUMMARY

Rabbits immunized with egg albumin produce a homocytotropic antibody. The antibody is identified by its ability to produce passive anaphylaxis in rabbit skin. The time of appearance of this antibody, its persistence and recall after booster injections depends, in part, on the route of immunization and the adjuvant employed. The physicochemical characteristics of the homocytotropic antibody obtained was similar regardless of the immunization schedule used. The anaphylactic activity of these antisera showed some heterogeneity when chromatographed on diethylaminoethyl (DEAE)-cellulose, but all fractions were inactivated by heating and absorption with a specific antisera. The anaphylactic activity could be separated from rabbit IgG and IgA, and was not blocked by absorption with antisera specific for these classes of immunoglobulins. Anaphylactic activity was completely removed by absorption with a specific antiserum which did not react with any of the known rabbit immunoglobulins. The passive cutaneous anaphylaxis titer of a rabbit serum containing homocytotropic antibody was reduced by 50% after absorption with an antisera (anti-FcND) specific for human IgE. On the basis of these distinctive physicochemical characteristics, it is concluded that rabbit homocytotropic antibody represents a unique class of rabbit immunoglobulin, analogous to human IgE.

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