

Association of rabbit reaginic antibody with an immunoglobulin distinct from IgG, IgA, and IgM

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Reaginic antibody was demonstrated in the serum, colostrum, and milk of rabbits which had been immunized during pregnancy with dinitrophenylated bovine serum albumin. The levels of the reaginic antibodies to DNP did not parallel those of IgM agglutinating antibodies in serum or IgA antibodies in colostrum, and their chromatographic behaviors on Sephadex G-200 and DEAE cellulose were also different from those of IgA, IgG, and IgM. Further evidence that the rabbit reagins belong to a unique class of immunoglobulins was obtained by the demonstration that the reagins were absorbed by anti-light chain serum but not by anti- γ -, anti- μ -, or anti- α -chain sera.

In response to parasitic infection or immunization with various antigens, rabbits may form antibodies with the capacity to sensitize homologous skin for passive cutaneous anaphylaxis.¹⁻³ These antibodies, referred to as reagins in the present work, have been shown to possess physicochemical properties different from IgG and IgM^{2, 3} and have been claimed to be associated with the 7S IgA.³ In view of the recent findings relating human reagins to a new immunoglobulin, IgE,⁴ it appeared possible that rabbit reagin might belong to a class of immunoglobulin other than IgG, IgM, or IgA. In the present work experiments designed to investigate this possibility are described. Rabbit colostrum, a source rich in IgA,^{5, 6} was used to facilitate studies on the relationship between reagin and IgA.

MATERIALS AND METHODS

Animals

Random-bred female New Zealand white rabbits weighing 2 to 3 kilograms were used for immunizations and skin tests.

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Antigen

Bovine gamma globulin (BGG) and crystalline bovine serum albumin (BSA) were obtained from Armour Pharmaceutical Co. and conjugated with dinitrofluorobenzene (Mann Research Lab. New York) according to the method of Levy.⁷ The average number of 2,4-dinitrophenyl (DNP) groups per molecule of carrier protein was 9 for DNP-BSA and 12 for DNP-BGG.

Antisera

The goat anti-rabbit light-chain serum was donated by Dr. Z. Lummus, and the goat antisera against rabbit α , γ , or μ heavy polypeptide chains were provided by Dr. J. Cebra. Anti- μ chain was prepared by immunizing goats with purified anti-salmonella IgM-antibody. Anti- α chain was prepared by immunization with homogeneous IgA-immunoglobulin isolated from rabbit colostrum, and anti- γ chain was prepared by immunization with papain digest fragment III of purified rabbit IgG. Bis-diazobenzidine cross-linked insoluble rabbit IgG was used to absorb the nonclass-specific antibody present in anti-M and anti- α . Methods for the preparation and immunochemical characterizations of these reagents have been reported,⁶ and their application to immunocytological studies has been documented elsewhere.⁸ The γ -globulin fractions of the reagents were used in the absorption studies.

Immunization

Pregnant rabbits were injected in the hind footpads with 10 mg. DNP-BSA at different times prior to delivery. The DNP-BSA was either in an alum precipitate or emulsified in complete Freund's adjuvant. The animals were bled daily, beginning 5 days after immunization. The newborn rabbits were exsanguinated at birth and the sera were pooled from each litter. Sera were stored in small aliquots at -20° C. before being used.

Colostrum

Rabbits were milked following parturition and daily thereafter. The colostrum was diluted with a half volume of 0.15M NaCl and centrifuged at 15,000 r.p.m. for one hour at 4° C. After centrifugation the clear fluid between the floating fatty layer and the pellet was withdrawn with a syringe.

Passive hemagglutination

DNP-BGG was used to coat tannic-acid-treated sheep erythrocytes according to the method of Stavitsky.⁹ Serum titrations were carried out in microtiter trays. 2-Mercaptoethanol-sensitive antibody was measured before and after treatment of serum with an equal volume of 0.2M mercaptoethanol (2-ME) for 2 hours at room temperature.

Homologous passive cutaneous anaphylaxis (PCA)

Reaginic antibodies were demonstrated by a homologous passive cutaneous anaphylaxis method. Normal rabbits were injected intradermally in the shaved backs with 0.2 ml. of the test samples. Three days later antigenic challenge was

performed by injection of 20 μ g DNP-BGG into the previously prepared sites and intravenous injection of 1.5 ml. of 2 per cent Evans blue dye in saline. The results were recorded 45 minutes after challenge. Bluening of an area measuring 3 mm. or more in diameter on the outer surface of the skin was regarded as a positive reaction. Blocking tests were carried out by injection at the same sites of 0.2 ml. of the test samples 24 hours prior to injection of a standardized reagin-containing serum. This was followed 3 days later by antigenic challenge.

Sephadex gel filtration and ion-exchange chromatography

Five milliliters of clarified colostrum was applied to a column of G-200 Sephadex (2.5 cm. $\times$ 90 cm.) equilibrated with 0.1M phosphate buffer, pH 6.8. Gel filtration and subsequent chromatography were carried out at 4° C. Fractions obtained from the gel-filtration column were pooled and concentrated by pressure dialysis against 0.1M phosphate buffer, pH 6.8. The concentrated material was applied to a column of diethylaminoethyl (DEAE)-cellulose equilibrated in 0.01M phosphate buffer, pH 7.5. After collection of a column volume of effluent, stepwise elutions with 0.05M, 0.1M, 0.2M, 0.3M, 0.4M, and 1.0M NaCl in starting buffer were carried out consecutively as described by Cebra and Robbins.⁶ Testing of the fractions for antibody activity was carried out either before or after concentration by pressure dialysis.

Absorption with specific antisera

Two reagin-containing sera were used, one containing anti-BSA reagins to a titer of 1:8 and one containing anti-DNP reagins in the same titer. For the absorption, the reagin-containing serum was mixed with an equal volume of anti-serum. A γ -globulin preparation of normal goat serum was used as control. After incubation for one hour at 37° C. and thereafter overnight at 4° C., the mixtures were centrifuged and the supernatant fractions were tested for reaginic antibody

Table 1. *Agglutinating and reaginic antibodies in serum and colostrum*

Rabbit No.	Days after antigen injection	Serum		Colostrum		Newborn serum	
		Agglutinin	Reagin	Agglutinin	Reagin	Agglutinin	Reagin
17	6	64*	-	8	-	N.D.	N.D.
25	7	128	+	16	$\pm$	64	$\pm$
14†	9	16	-	4	-	16	-
351	9	64	+	2	+	< 2	-
26	10	256	+	32	$\pm$	N.D.	$\pm$
352	10	128	-	16	$\pm$	8	-
353	13	128	-	32	-	16	-
23	27	4	-	< 2	-	2	-

Antibody tests were performed on sera obtained on the day of delivery and on colostrum samples obtained one day after delivery.

*Numbers refer to reciprocal of the highest sample dilution showing agglutinating antibody activity; N.D., not done.

†Rabbit No. 14 developed a late reagin response with maximum serum reagin titer attained 3 weeks after immunization; at that time anti-DNP reagins were also demonstrable in the milk.

activity. To determine whether the absorptions were complete, the supernatant fractions were also tested for the presence of excess antisera by gel diffusion.

RESULTS

Eight pregnant rabbits were immunized with 10 mg. DNP-BSA. Seven of these responded with anti-DNP serum-reagin formation. The serum reagins were demonstrable on the sixth day after immunization in 5 rabbits and on the ninth and tenth days in another 2 rabbits. The reagins reached peak titers at an average of 8 to 9 days after immunization and disappeared from the circulation rapidly thereafter. Reagins were also demonstrable in colostrum from rabbits with serum-reagin response (Table I). The levels of colostral reagins were low, not exceeding a titer of 1:2, and demonstrable only in colostrum from rabbits which showed or had shown a serum-reagin titer of, or greater than, 1:8. The reaginic activity found in serum as well as in colostrum was sensitive to treatment with mercaptoethanol and likewise was destroyed after heating at 56° C. for 4 hours. In one rabbit, reagins were demonstrable in milk obtained 9 and 10 days after delivery at a time when peak serum-reagin response was encountered.

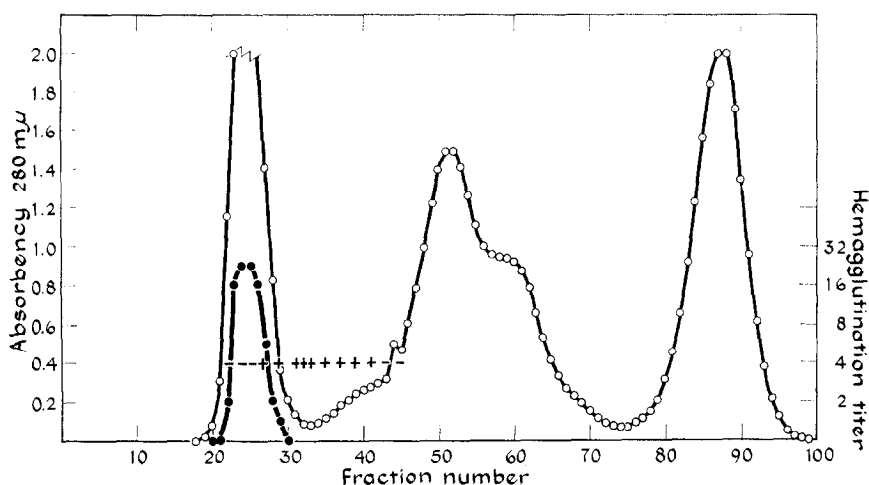
Agglutinating antibodies, sensitive to 2-ME, appeared simultaneously in serum and colostrum. Analysis of these antibodies by Sephadex G-200 chromatography and with specific anti-heavy chain sera revealed the serum antibodies to be of the IgM type and the colostral antibodies to be of the IgA type. In 4 cases reagins were demonstrated in serum one day before the appearance of agglutinating antibodies. There was no correlation between titers of colostral IgA agglutinating antibodies and occurrence of reagins (Table I).

Each of the pools of sera from the newborn rabbits contained anti-DNP agglutinins, and the titers were either equal to or lower than those of the maternal sera. The newborn sera were faintly positive in homologous PCA reactions in two cases, and in both, the mother had shown a reagin response of titers of or greater than 1:8.

Chromatographic separation of colostrum

Following gel filtration of a sample of reagin-containing colostrum on Sephadex G-200, the reagins were eluted with maximum activity between the first two major peaks. Analysis of concentrated eluates with specific anti- γ -, and anti- μ -, and anti- α -chain reagents showed that the first peak contained predominantly IgA, and the next peak, appearing as a shoulder on the ascending side of the second major peak, contained mainly IgG. Gel filtration of another colostrum sample, which contained 2-ME sensitive agglutinating antibodies but no anti-DNP or anti-BSA reagins, resulted in elution of all the agglutinating antibodies in the IgA peak. Fractions from this separation were then used to localize reaginic anti-BSA blocking activity. In accordance with the result of the gel-filtration experiment with reagin-containing colostrum described previously, fractions eluted in the region between the first two peaks by G-200 Sephadex filtration were shown to be able to block the reaginic activity of an anti-BSA rabbit serum (Fig. 1).

Further separation by DEAE chromatography of the IgA peak obtained by

**Fig. 1**

G-200 Sephadex filtration of colostrum obtained from a rabbit immunized with DNP-BSA 13 days before delivery. ○—○, OD 280 mμ; ●—●, hemagglutination with DNP-BGG coated erythrocytes; (+) presence and (−) absence of reagent anti-BSA antibody-blocking activity. In another experiment, colostral reagent antibodies to DNP were eluted in that portion of the elution pattern corresponding to the blocking activity indicated in the figure.

gel filtration gave two major fractions eluted with 0.01M phosphate buffers, pH 7.5, containing 0.2M and 0.3M NaCl, respectively. Both peaks contained antigenically identical IgA as judged by results of gel diffusion against specific goat anti-α serum. The agglutinating antibodies were eluted only in the first (0.2M) peak, whereas the reagent-blocking activity was recovered only in the second (0.3M) peak.

Absorption with specific anti-heavy and anti-light chain reagents

Goat anti-rabbit reagent globulins specific for either light chains or heavy chains (α, μ, and γ) were used for the absorption studies. None of the specific anti-heavy chain reagents used absorbed the reagents (Fig. 2). However, a total removal of reagent antibody was observed after absorption with anti-light chain. The anti-IgA and anti-IgM used for the preparation of anti-α and anti-μ reagents by treatment with insolubilized rabbit IgG were also capable of absorbing the reagents. Removal of anti-light chain antibodies in these antisera with the insolubilized IgG abolished their ability to react with reagents.

DISCUSSION

The results of the present study indicate that rabbit reagent antibody belongs to a class of immunoglobulins distinct from IgG, IgA, and IgM. This conclusion is based upon the following findings:

1. The titers of reagent antibody to DNP in colostrum did not parallel those of the IgA agglutinating antibodies in this body secretion.

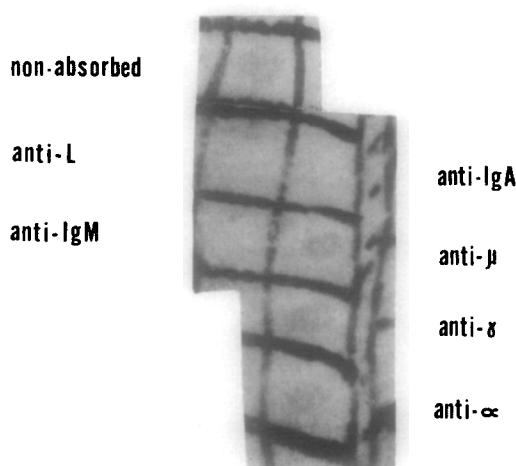


Fig. 2

Homologous PCA reactions with supernatant fractions obtained after the absorption of rabbit serum containing anti-DNP reagins with respective goat anti-rabbit reagents. Three days after injection of the absorbed samples into skin sites, antigenic challenge was performed by injecting 20 μ g DNP-BGG into each of the prepared sites. The goat anti-rabbit reagents used were specific for either light chains (L) or for heavy chains (α , γ , and μ) or directed against whole immunoglobulins (IgA and IgM).

2. The presence of serum reagins did not appear to correlate with the titers of serum IgM agglutinating antibodies.

3. The elution patterns of reaginic antibody obtained by gel filtration and DEAE chromatography suggest that it has a molecular weight between 7S and 19S and is distinct from colostral IgA and IgG.

4. Absorption studies with specific antisera indicate that rabbit reaginic antibody possesses light chains but not the heavy chains specific for IgG, IgA, or IgM.

In a previous report on the nature of reaginic antibody, Onoue and associates³ suggested that rabbit reagins are of IgA nature. Since absorption experiments with specific anti- α chain were not carried out in their studies, it is possible that the reaginic activity ascribed to 7S IgA was in fact due to impurities in the IgA fraction.

According to Zvaifler and Becker,² the properties of rabbit reagins include persistence of fixed antibodies in homologous skin, susceptibility to heat and 2-ME, γ_1 electrophoretic mobility, and a molecular size intermediate between IgM and IgG. These properties are similar to those of human reaginic antibody.¹⁰ Our studies indicate that the rabbit reagins may belong to a distinct immunoglobulin class which may be comparable to IgE, now considered to be the immunoglobulin class responsible for reaginic activity in the human being.¹¹

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