STUDIES ON THE RELATIONSHIP OF ALLOTYPIC SPECIFICITIES TO ANTIBODY SPECIFICITIES IN THE RABBIT*

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The proteins whose allotypic variability has been most closely studied up to the present time are the rabbit gamma globulins. Their function as antibodies has been utilized from the beginning of these studies since the isoimmunizations which led to the discovery of allotypy were made by injection of specific precipitates (1–3). One could thus ask if there exist qualitative or quantitative differences related to allotypy among rabbit antibodies against different antigens. It was even more natural to pose this question since other workers had observed among antibodies against different antigens, differences of another nature, revealed by chromatographic analysis on CM cellulose (4) or by starch gel electrophoresis after reduction and alkylation in 8 M urea (5). In order to answer the above question, rabbits were subjected to successive immunizations with different antigens,—hen egg albumin (OA), pneumococcus Type II, and dinitrophenol (DNP)–bovine γ-globulin. The antibodies from each rabbit against different antigens were purified from specific precipitates. The antibody preparations from each rabbit were reacted with antisera against the allotypic specificities found in that rabbit, and the reactions were compared.

Materials and Methods

Hen ovalbumin (3 times recrystallized) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Purified Type II pneumococcal polysaccharide was kindly provided by Dr. Charles Todd and had been prepared according to the method of Heidelberger, Kendall, and Scherp (6) from cultures kindly supplied by Dr. Wahl. Bovine γ-globulin was prepared by precipitation from serum by 33 per cent saturation with $(NH_4)_2SO_4$ and further purified by passage through a diethylaminoethyl cellulose column equilibrated with PO_4Na, 0.01 M, pH 7.4. DNP–bovine γ-globulin containing approximately 40 mols DNP per 160,000 *g* of bovine γ-globulin was prepared by a modification of the method of Eisen, Carsten, and Belman (7). Equal weights of γ-globulin and 2,4-dinitrobenzene sulfonic acid were reacted in 2 per cent K_2CO_3 followed by extensive dialysis against 1 per cent KCl.

Six rabbits selected for maximum heterozygosity in their allotypic formulas were immunized simultaneously with approximately 10 mg each of DNP–bovine γ-globulin and hen ovalbumin in complete Freund's adjuvant. After 1 month the animals were bled and reinjected; 1 month

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later, the animals were bled every few days for several weeks. This schedule was repeated until 100 to 150 ml of serum had been harvested from each animal. For rabbit B-142, two separate batches of serum containing antiovalbumin antibody were obtained. The first batch was a mixture of sera obtained during the first 3 months of immunization and the second batch during the 4th and 5th months of immunization. At this point, the animals were subjected to a series of intravenous and subcutaneous injections over a 3 week period with a suspension of formalinized Type II pneumococci. Each animal received an antigen dose containing a total of 560 μg of nitrogen. Serum was then harvested until 100 to 125 ml had been taken from each animal.

The animals with the best antibody responses were chosen and purified antibody was prepared from specific precipitates utilizing the techniques described by Heidelberger and Kabat (8) for antibodies against pneumococcal polysaccharide, by Singer, Fothergill, and Shainoff (9) for anti-OA antibodies, and by Farah, Kern, and Eisen (10) for anti-DNP antibodies. Although the amounts of antibody thus obtained in the various preparations were unequal and often small, it was sometimes possible to determine the proportion of protein precipitable by antigen in a given preparation. This proportion was 63 per cent for the anti-DNP preparation of rabbit B-126, and 83 per cent for the anti-DNP preparation of rabbit B-160 (determinations made according to the method of Farah, Kern, and Eisen, reference 10, utilizing optical density measurements at 278 mμ and 360 mμ on the supernatants from the quantitative precipitin reaction); 86 per cent for the anti-OA preparation from rabbit B-160 (determinations made using Nessler's reagent); 91 per cent for the 1st anti-OA preparation from rabbit B-142 (optical density at 278 mμ) and 100% for the 2nd anti-OA preparation (Nessler's reagent).

The antibodies thus purified from the antisera of the three rabbits were brought to a suitable protein concentration (optical density measurements at 278 mμ: 3.39 for the antibodies of rabbit B-126 and B-160, 4.86 for the antibodies of rabbit B-142, corresponding to a protein concentration of the order of 2.3 to 3.5 mg/ml). Optical density measurements on the anti-DNP antibody preparations were made at 278 mμ and 360 mμ in order to determine the absorption contributed by any small quantity of DNP or DNP–bovine γ-globulin remaining in the preparation. This permitted one to determine that, of the total absorption at 278 mμ, not more than 0.6 per cent was contributed by any free DNP present and not more than 1 per cent by any DNP–bovine γ-globulin in the preparation from rabbit B-160, and in the case of rabbit B-126, 1.5 and 2.5 per cent respectively. The antibody preparations were reacted with rabbit antisera against the different allotypic specificities found in the serum of the experimental animals before immunization. At the same time, each of the antisera against the allotypic specificities A1, A2, A4, A5[1] was reacted against a gamma globulin solution prepared by ammonium sulfate precipitation (33 per cent saturation) from a sample of antiserum from each animal.

These reactions were carried out in gel medium (simple diffusion, in tubes whose internal diameter was about 1.7 mm). The total volume of serum (immune and non-immune) contained in the lower gel layer was, in all the tubes, equal to half the volume of that layer, so that the total concentration of proteins was, in all the tubes, significantly higher in the gel layer of antiserum than in the liquid layer of antigen. The tubes were photographed at the end of 7 days. The penetrations of the zones of precipitation, that is the distances between their leading

[1] The allotypic specificities previously designated in this laboratory by *b*, *c*, *d*, and apparently determined by allelic genes are designated by A1, A2, A3. The specificities apparently directed by another series of alleles, and previously designated by *a*, *g*, *f*, are designated respectively by A3, A4, A5. Sera, molecules, or rabbits will be referred to as $A1^+$, $A2^+ \cdots$ to designate the molecules which carry the given specificity or the rabbits whose sera contain these molecules (3).

edges and the interface, were measured on the negatives whose images were magnified 2 times. Although the leading edges of the zones of precipitation were, in these reactions, less precise than usual with protein antigens, the errors which could have resulted from the imprecision are certainly of an order of magnitude smaller than the concentration differences which will be discussed below. Each of the four antisera against the different allotypic specificities was reacted, under the same conditions as with the solutions of purified antibody, with dilutions of a solution of gamma globulin containing the given specificity. These reactions served to establish a reference curve for each of the specicifities A1, A2, A4, A5. The penetrations found in the reactions of the different solutions were compared to this curve. The numbers read from the abscissae could be considered as proportional to the concentration of molecules which carried the given specificity, assuming that these molecules had, in the solution studied and in the standard solution, the same diffusion coefficient and the same combining ratio with the antibody.

The evaluation of the relative concentrations of the molecules bearing the A3 specificity was made in another manner because of the cloudy character of the precipitation zone in the reactions of the anti-A3 sera which were available. Variable quantities of each preparation of antibody from the two $A3^+$ rabbits, in a constant volume, were mixed in liquid medium with a constant volume of anti-A3 serum and centrifuged. A determination was made on the supernatants to find the two dilutions of the preparation of antibody between which occurred the amount that caused the disappearance of ability of the supernatant to react with a nonimmune $A3^+$ serum. The relative concentration of $A3^+$ molecules in an antibody preparation is between the reciprocals of these two dilutions.

RESULTS

Let x, y$\cdots$ be allotypic specificities and Cx(γ), Cx(OA)$\cdots$ the concentrations of molecules which carry these specificities in the preparations of γ-globulin, anti-OA antibody$\cdots$ in the serum of a given rabbit. It was not possible, in the present work, to determine the absolute values of these concentrations, nor consequently was it possible to compare the absolute values of Cx(γ) and Cy(γ) or of Cx(OA) and Cy(OA). In addition, the solutions of γ-globulin prepared by ammonium sulfate precipitation contained an unknown quantity of other proteins and the total protein concentrations of the different solutions (which, as has been indicated, were often low) were determined by optical density measurements at 278 mμ which allowed the possibility of errors. Thus the concentrations of these solutions are only approximate although there is no question as to their order of magnitude.

Table I gives for each of the allotypic specificities, A4, A5, A1, A2, and for each of the antibody preparations, the value found for the ratio of the concentration of molecules which carry a given allotypic specificity in a given preparation to the concentration of molecules which carry the same specificity in the γ-globulin of the same animal.

DISCUSSION

Each of the figures in Table I is a ratio of two values whose significance has been discussed above, and is subject to the same reservations noted above.

However, if in a given preparation of antibody, the concentration of molecules which carry the different allotypic specificities were in the same proportion as in the γ-globulin fraction, the figures shown in the same line would be equal (they would be larger or smaller than 1 depending on whether the concentrations were higher or smaller than in the γ-globulin fraction).

One thing to be noticed in Table I is the frequently great inequality among the figures which, on the same line, concern the same animal. Another observation is the usual absence of parallelism between two different lines of figures.

TABLE I

*Relative Concentrations of the Molecules Carrying the Different Allotypic Specificities in the Different Preparations of Antibodies**

Animals	B-126			B-160			B-142			
Allotypic specificities	A4	A5	A1	A4	A2	A3‡	A4	A5	A1	A3‡
Antibody preparations										
Anti-OA I	1.0	2.2	1.05	1.35	1.25	>40 <80	1.3	1.1	1.4	>22 <30
Anti-OA II	—	—	—	—	—	—	1.3	1.0	1.4	>22 <30
Anti-DNP	1.2	0.4	0.85	1.35	0.35	>40 <80	0.85	1.55	0.35	>30 <45
Anti-Type II pneumococcal polysaccharide	0.2	0.55	0.3	0.55	1.3	>7 <10	—	—	—	—

* The concentration unit, differing according to the animal, is, for the specificities A4, A5, A1, and A2, the concentration of molecules carrying the same specificity in a preparation of gamma globulin of the same animal.

‡ The limits indicated for the concentration of A3 have been determined by reactions in liquid medium, for only the antibody preparations and not for the gamma globulin preparation; the numbers have only a comparative significance.

The inequality among the figures of one line signifies that, in almost all the antibody preparations, the concentrations of molecules carrying the different allotypic specificties are in different proportions from those in the γ-globulin solution of the same animal. The absence of parallelism between two different lines of figures signifies that the proportions of molecules bearing two different allotypic specificities are usually not the same in two preparations of antibodies from the same animal against different antigens. In the single case of 2 solutions of antibody from the same animal against the same antigen (anti-OA antibody preparation I and II made from rabbit B-142 from two batches of serum collected at different times during the course of immunization) the concentrations are, on the contrary, similar and the sole discordance observed hardly exceeds the experimental errors, considering what has been said of the frequent lack

of sharpness of the leading edges of the precipitation zones in the reactions of rabbit antiallotype sera.

The differences observed among several preparations of antibodies against different antigens are only, however, differences of proportion: all the specificities found in the serum of an animal have been found in all the antibody solutions prepared from the serum of the same animal. The concentration of molecules bearing these specificities, although at times relatively small, was always sufficient to give a definitely positive precipitation reaction at the interface in liquid media. Results obtained by other workers, having some similarity to the present work, came to our attention during the editing of the present article. Gell and Kelus (11) have observed the elimination of one and perhaps two allotypic specificities (A5 and A3) in antibodies against a hapten, or more exactly in the solution of specific precipitate in the presence of excess hapten. Such a complete elimination of an allotypic specificity from antibody of a given specificity was not observed here, and indeed Gell and Kelus have reservations about completeness of such elimination in their experiments. In addition, according to G. W. Stemke, the addition of an excess of anti-A5 antiserum to anti-T2 phage antiserum from $A5^+$ rabbits, resulted in either a considerable diminution of anti-T2 titer or did not appreciably modify this titer, depending on the individual rabbit (12).

One might wonder whether the differences in proportions which we have observed can be attributed to a relation between the allotypic specificities and the antibody specificities of the same protein molecules. If one designates the figures in Table I by C4(OA), C5(OA)$\cdots$ C4(DNP)$\cdots$ one notices, for example that for rabbit B-126, C4(OA)/C5(OA) = 1.0/2.2 and C4(DNP)/C5(DNP) = 1.2/0.4. One might be tempted to conclude from this that among the $A4^+$ and $A5^+$ molecules, that is those carrying the A4 and A5 specificities (determined by allelic genes and so far not found on the same molecule; references 13 and 14), the $A5^+$ molecules are more apt than the $A4^+$ molecules to carry the antiovalbumin antibody specificity, while the $A4^+$ molecules are more apt to carry the anti-DNP antibody specificity. However, this conclusion is not in accord with the comparison of the concentration ratios found with the other rabbit which also carried both A4 and A5 specificities. With this rabbit (B-142), it is noticed that C4(OA)/C5(OA) = 1.3/1.1 and C4(DNP)/C5(DNP) = 0.85/1.55.

If one supposes that the synthesis of molecules, or of parts of molecules, each of which bears 1 of the 2 specificities directed by allelic genes, is directly dependent on these 2 genes and that they are a part of the chromosomic complement of the same cells, one would expect that the molecules bearing these two specificities would be synthesized by each cell, and consequently, in a constant ratio.[2] There is no evident reason why the cells which may be special-

[2] There is, however, some recent evidence that in a diploid cell in mammalian females, only one of the two X chromosomes is active (15).

ized in the formation of antibodies of a certain type should act differently. However, it is seen in Table I that the proportion of molecules carrying two allelic specificities such as A4 and A5, A1 and A3, A2 and A3 vary from one preparation of antibody to another. This variation would be more easily understood if one supposed that the molecules which carry, respectively, two different allelic specificities were not synthesized by the same cells. A similar hypothesis has recently been put forward by Harboe, Osterland, Mannik, and Kunkel (16) to explain the observation that in a human heterozygote, the myeloma globulins, supposedly formed by a single cellular clone, carry only one of two specificities directed by allelic genes. This hypothesis seems also in accord with recent results of Dray who showed, in young rabbits whose mother had been immunized against a given allotypic specificity, a diminution of the concentration of molecules bearing the given specificity but no diminution in those bearing the allelic specificity (17). This hypothesis is, however, not in agreement with very recent experiments (18): it was observed by the use of fluorescent antibodies that, in a heterozygous rabbit, the allelic specificities A4 and A5 were always found together in the cytoplasm of the same lymph node cells.

SUMMARY

Purified antibodies against three different antigens and haptens (ovalbumin, dinitrophenol, and Type II pneumococcal polysaccharide) were prepared from specific precipitates from the antisera of several rabbits. The reactions of these preparations with antisera against the allotypic specificities carried by each of the animals forming the antibodies were used to compare the relative concentrations of molecules bearing each specificity in the different solutions of antibodies from each rabbit. These relative concentrations appeared to vary greatly from one preparation to another, although all the allotypic specificities carried by each animal were found in all the preparations of antibodies from that animal. The interpretation of these results is discussed.

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