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Similar Idiotypic Specificities in Immunoglobulin Fractions with Different Antibody Functions or Even without Detectable Antibody Function

(ovalbumin/immunoadsorption)

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ABSTRACT **Idiotypy has been studied in antibodies against a protein antigen: hen ovalbumin. Various fractions of antisera to hen ovalbumin have been compared from the standpoint of the idiotypic specificities found on the immunoglobulins that they contain. Idiotypic specificities were found to be common to (*a*) antibodies that were not eluted from the same immunoadsorbent by the same $MgCl_2$ concentration, but by four different concentrations. (*b*) Antibodies that were precipitable only by hen ovalbumin, only by hen and turkey ovalbumin, or also by duck ovalbumin. (*c*) Antibodies that were precipitated or not precipitated by the homologous ovalbumin, and proteins apparently devoid of antiovalbumin antibody function. These findings are discussed from the standpoint of (*i*) the differences in function between the various fractions with the same idiotypic specificities, and (*ii*) what may be common in the cellular origin of immunoglobulins with a common idiotypic specificity, and what may be different in immunoglobulins with different antibody functions or without antibody function.**

The same idiotypic specificities have been found in certain IgG and IgM antiovalbumin antibodies.

Idiotypy of antibodies has been defined as their property of possessing different antigenic specificities, both among antibodies of one individual against distinct antigens and among antibodies of different individuals (or perhaps of different groups of individuals) against the same antigen (1, 2). Since the time when the first observations of this property were made, almost simultaneously for human (3) and rabbit (4) antibodies, further observations have been made in rabbits, concerning antibodies against antigens that were found to be carbohydrate in nature, when this nature was known, and also against haptenic groups (5–16).

It might be inferred from the structures of proteins and polysaccharides that the variety of antigenic determinants would be greater in proteins than in polysaccharides; this might explain the frequent crossreactivity observed among phylogenetically unrelated polysaccharides, but not among proteins. It therefore seemed of interest to study also idiotypy among antiprotein antibodies. Ovalbumin was chosen as the first protein antigen to be studied, and this paper describes work designed to compare idiotypy among antibodies that have properties different from each other.

Abbreviations: Ov, ovalbumin; PCA, passive cutaneous anaphylaxis; PA fraction, precipitating antibodies; NPA fraction, nonprecipitating antibodies; NRP fraction, nonreacting protein.

MATERIALS AND METHODS

Preparation of Ovalbumins and Antisera to Ovalbumin. Hen ovalbumin (hen Ov) and turkey ovalbumin (turkey Ov) were crystallized five times and three times, respectively, by salting out with $(NH_4)_2SO_4$, and each crystal preparation was washed twice. Duck ovalbumin (duck Ov) was precipitated twice by 60%-saturated $(NH_4)_2SO_4$ and washed three times after each precipitation. The reactions of each of the three ovalbumin preparations (concentration 1–2 mg/ml) with the antisera to hen Ov in gels resulted in a single precipitation zone.

Antisera to hen Ov were prepared in the rabbit by intravenous injections (4 per week during several months) of 1–6 mg of hen Ov with alum.

Antiidiotypic Immunizations. For the purpose of antiidiotypic immunizations, antibodies to hen Ov were prepared from antisera to hen Ov of two rabbits (No. 663 and 765). The precipitates, in proportions close to equivalence of each of these two antisera with hen Ov, were washed three times in normal saline, dissolved in a veronal acetate buffer (pH 2), and a suspension of $BaSO_4$ in this solution was made, then centrifuged (17).

The antibodies of the two antisera to hen Ov were used, respectively, in two series of antiidiotypic immunizations (6 rabbits in each series). It could be speculated that the first antiidiotypic immunizations against anti-*Salmonella typhi* antibodies (by injections of agglutinated bacteria) were favored by the presence of the somatic antigen of *S. typhi*, which is an adjuvant (18). The rabbits used in these two series of immunization were given intraperitoneal and intravenous injections of mixtures of purified antibodies (25–250 μg per injection) with alum, and with one tenth of these doses of somatic *S. typhi* antigen; the schedule of these injections is the same as that adopted for immunization with agglutinates of *S. typhi* (4).

Preparation of Fractions of Antibodies to hen Ov for the Comparison of Their Idiotypic Specificities. The immunoadsorbents used in these fractionations were prepared by the reaction of glutaraldehyde with hen, turkey, and duck Ov (19) and, in one case, by linkage of hen Ov with *p*-aminobenzylcellulose at pH 8 (20). After the immunoadsorbents had combined with the antibodies and had been washed, the antibodies were eluted by $MgCl_2$ solutions (19) at the concentrations indicated below (*Results*), or by a 0.5 M glycine·HCl buffer (pH 1). The precipitating antibodies of the antiserum

to hen Ov (No. 663) were prepared from a precipitate of this antiserum with hen Ov in proportions close to the equivalence, by solution (after washing three times with normal saline) in 0.5 M glycine·HCl buffer (pH 2), and fractionation of the solution on a Sephadex G-200 column equilibrated with the same buffer (21).

Antigen–Antibody Reaction. The sera from all bleedings of rabbits that received antiidiotypic immunizations were reacted with the homologous antiserum to hen Ov, first, merely in interfacial reactions, in liquid media (so called ring-tests), and subsequently in gels (22), by the technique of double diffusion in tubes.

The comparative precipitation reactions, in gels, of a selected antiidiotypic serum with several samples or fractions of antiserum to hen Ov were performed according to the technique of double diffusion in one dimension, in cells with parallel walls (23).

Sheep erythrocytes combined with hen Ov by means of glutaraldehyde (24) were used for the hemagglutination tests. Signs of an antigen–antibody reaction were also investigated by means of passive cutaneous anaphylaxis (PCA) (25).

RESULTS

The sera of a proportion of each of two groups of the rabbits (2 and 4 out of 6 in each series) that received two series of antiidiotypic immunizations formed a precipitate with the antisera to hen Ov from which the antibodies used in the immunization had been, respectively, prepared; they did not form a precipitate with the serum sample taken from the same rabbits before immunization with hen Ov. It is known that this feature is peculiar to idiotypy and contrasts with allotypy (4, 8, 9). The most potent precipitating-serum sample from each series of immunized rabbits did not form a precipitate with (interfacial reaction in liquid medium) any of the antisera to hen Ov of 27 rabbits, other than that which was the source of the immunizing antibodies to hen Ov.

The idiotypic specificities detected in antisera to hen Ov were compared with various immunoglobulin fractions prepared from these sera in three different ways.

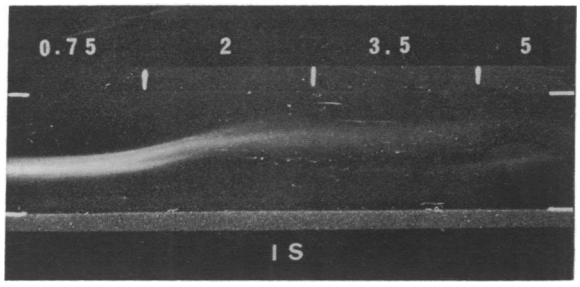

FIG. 1. Reaction, in an agar gel, in a cell with parallel walls (double diffusion in one dimension) of an antiidiotypic rabbit serum (no. 793) prepared against antibodies to hen Ov (*IS*, lower layer) with four fractions of antibodies to hen Ov of rabbit No. 663, eluted from the hen-Ov-immunoadsorbent column by 0.75, 2, 3.5, and 5 M $MgCl_2$ solutions, at the concentrations of 1.3, 1, 1.3, and 2 mg/ml, respectively (*upper layers*). The white dashes indicate the interfaces between the various layers. Among the three idiotypes shown by the three precipitation zones that can be distinguished, at least two are present (in different proportions) in all four antibody fractions.

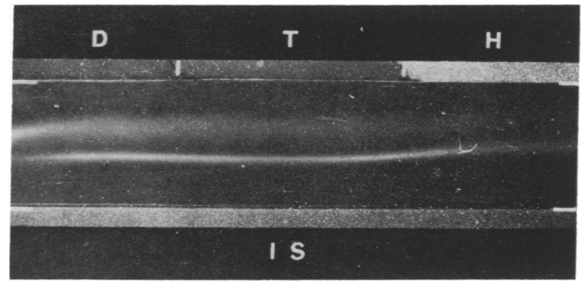

FIG. 2. Reaction, in conditions similar to those of Fig. 1, of the antiidiotypic serum No. 793 (*IS*, lower layer of the cell) with three antibody fractions of antiserum to hen Ov No. 663. These are: *D*, adsorbed on, then eluted from immunoadsorbent of duck Ov; *T*, the filtrate of *D* adsorbed on, then eluted from turkey Ov; *H*, the filtrate of T adsorbed on, then eluted from hen Ov. The concentrations were D, 2.5; T, 1.9, and H, 0.9 mg/ml. The two distinct and almost parallel precipitation zones in front of the three upper fractions show that two idiotypes are present in all three fractions, in spite of the different properties of the antibodies to hen Ov of which they are made.

(a) Antibody fractions combined with hen Ov immunoadsorbents and eluted by $MgCl_2$ at various concentrations

The concentrations of the $MgCl_2$ solutions used for elution of the antibodies from a column of polymerized hen Ov were 0.75, 2, 3.5, and 5 M. It was established that the antibodies of these four fractions, adsorbed again on the same column of polymerized hen Ov, were totally eluted by an $MgCl_2$ solution at the same, but not at a lower, concentration. The reaction of these four serum fractions with the same anti-idiotypic serum is shown Fig. 1. It may be seen that at least two, and almost certainly three, idiotypes, which manifest themselves by as many precipitation zones, are present in all four fractions.

(b) Antibody fractions combined with immunoadsorbents of each of hen ovalbumin, turkey ovalbumin, and duck ovalbumin

By passage through a column of polymerized duck Ov, an antiserum to hen Ov (rabbit No. 663) was completely freed of antibodies that are able to combine with duck Ov. The absorbtion was considered complete when no appreciable amount of antibodies was eluted by 5 M $MgCl_2$ after the filtrate had been passed a second time through the same polymer. This second filtrate was then absorbed, in the same way, on a column of polymerized turkey Ov and, finally, on a column of polymerized hen Ov. The antibodies that combined with the three polymerized ovalbumins (duck, turkey, and hen Ov) were eluted by a solution of 5 M $MgCl_2$ to yield fractions duck, turkey, and hen, respectively.*

It can be seen from Fig. 2 that the same two idiotypes that are manifested by two quite distinct precipitation zones are present in all three fractions without any detectable difference

* Among the antibodies that combined with polymerized hen Ov, only 62% were precipitable by turkey Ov, but 97% combined with polymerized turkey Ov; thus, nearly all the antibodies to hen Ov that were not precipitable by turkey Ov were, however, able to combine with this heterologous antigen. This observation is in good agreement with an early observation that an excess of heterologous antigen (turkey Ov) inhibited the precipitation of an antiserum to hen Ov by hen Ov (26).

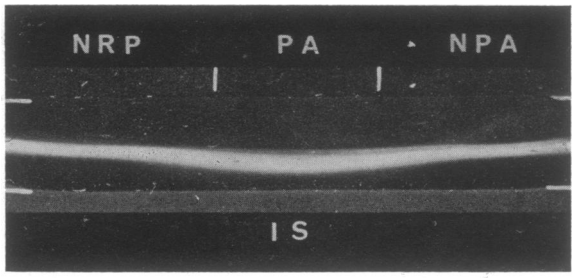

FIG. 3. Reaction, in conditions similar to those of Figs. 1 and 2, of the antiidiotypic serum No. 793 (*IS*, lower layer of the cell) with three fractions of antiserum to hen Ov of rabbit No. 663. Fraction *NRP* (0.45 mg/ml) is made of a protein that shows no sign of combining with hen Ov; fraction *PA* (2 mg/ml) is that of precipitating antibody recovered from the specific precipitate; fraction *NPA* (0.85 mg/ml) is that of antibody that did not precipitate with hen Ov but that did adsorb to polymerized hen Ov. Among the three precipitation zones (due to three idiotypes), which are visible on the right-hand side of the photograph, one is that of an idiotype present in fractions *NRP* and *PA*, but apparently not in *NPA*; the other two, which are denser and not clearly distinct on the left, are those of two idiotypes present in all three fractions.

of idiotypic specificity. Consistent results were obtained by a similar fractionation, on the same immunoadsorbents, of the antibodies of the other antiserum to hen Ov, No. 765, and the reaction of the fractions with a homologous antiidiotypic serum.

(c) Antibody fractions precipitable and nonprecipitable by hen Ov, and immunoglobulins without detectable antibody function

Precipitating antibodies were prepared as described above from a precipitate of 3 ml of antiserum to hen Ov with 2.3 mg of hen Ov (i.e., in slight antigen excess). Three peaks were observed when the precipitate L, dissolved in glycine·HCL buffers (pH 2), was passed through Sephadex G-200. The first corresponded to soluble compounds (partly precipitable after neutralization) and to macroglobulins; the second corresponded to IgG antibodies separated from the antigen, and the third corresponded to the antigen. The fractions of the second peak, fractionated again on Sephadex G-200 in order to remove the small amount of the other fractions that might have been mixed with them, constitute fraction PA (precipitating antibodies), which amounted to 3 mg.

The supernatant fluid after the precipitation of 30 ml of the same antiserum to hen Ov with hen Ov, which did not precipitate hen Ov but was still precipitable by the corresponding antiidiotypic sera, was fractionated on a Sephadex column [equilibrated with a 0.05 M phosphate buffer (pH 7)], and gave the three expected peaks. In order to separate as completely as possible the fractions of the second peak, which contained the uncombined IgG, from the soluble compounds contained in the fractions of the first peak, the fractions from the second peak were fractionated again on the same Sephadex column. These fractions were then passed through a column of immunoadsorbent that was made of polymerized hen Ov with which the nonprecipitating antibodies combined. These antibodies were eluted with a solution of 5 M $MgCl_2$. The eluate which, as expected, did not precipitate hen Ov solutions at the concentrations of 10–800 μg/ml, constitutes fraction NPA (nonprecipitating antibody), which amounted to 3.2 mg.

The filtrate was passed a second time through a column of polymerized hen Ov in order to ensure that it did not contain detectable amounts of antibody that is able to combine with this polymer, and can be eluted by 5 M $MgCl_2$. In order to make sure also that this filtrate did not contain antibodies to hen Ov that were directed against antigenic determinants destroyed by the action of glutaraldehyde, it was passed through a column of another immunoadsorbent of hen Ov–*p*-aminobenzyl-cellulose. Care was taken to prepare this immunoadsorbent in alkaline medium (pH 8), so that the protein would combine to the diazotized *p*-aminobenzyl-cellulose through its tyrosine groups. No detectable amount of protein was eluted from the washed column by a glycine·HCl buffer (pH 1). This filtrate was still precipitable by antiidiotypic sera. The material that, in this filtrate, combined with antiidiotypic antibodies was separated from the rest of the filtrate by passage through a column of immunoadsorbent made of an antiidiotypic serum (No. 793) that was polymerized by glutaraldehyde. Elution was performed with 5 M $MgCl_2$ and gave fraction NRP (nonreacting protein), which amounted to 1.1 mg. Fraction NRP was precipitable by an anti-rabbit IgG from sheep serum, and by rabbit antisera against the allotypic patterns of the IgG of the antiserum to hen Ov No. 663: Aa3 and Ab4.

The following investigations were made to make sure that fraction NRP contained no antibodies to hen Ov.

(*A*) Passive cutaneous anaphylaxis (PCA) was obtained with fractions PA and NPA, each at doses of 3.5 μg. No PCA was obtained with 100 μg of fraction NRP.

(*B*) Fractions PA and NPA agglutinated, at the concentration of 0.04 μg/ml, sheep erythrocytes combined with hen Ov through glutaraldehyde. Fraction NRP did not agglutinate the same hen Ov–erythrocytes, even at the concentration of 125 μg/ml.

Comparative reactions of fractions NRP, PA, and NPA with the antiidiotypic serum No. 793 are shown in Fig. 3. This reaction shows that at least one idiotype (and almost certainly two) is common to all three fractions; another idiotype present in fractions NRP and PA is apparently lacking in fraction NPA.

Idiotypic specificities common to antibodies of the IgG and IgM classes have been observed in antiovalbumin antibodies, as had been previously observed with antibodies against *S. typhi* (10).

DISCUSSION

Are the differences observed between the immunoglobulins contained in the various fractions that were separated by procedures *a*, *b*, and *c*, indicative of differences in antibody function in the sense of the specificity or affinity of antibodies.

(*i*) It has been verified by other authors with other precipitating systems that the antibodies which, after fixation on an immunoadsorbent, are eluted by different concentrations of the eluting agent, possess different immunochemical properties. For example, when antibodies to human-serum albumin that are fixed on a column of human-serum albumin–polystyrene were eluted by two successive buffers of pH 3 and 1, the antibodies against the "inhibitor fragment" of human-serum albumin were not eluted by the buffer of pH 3, but only by the more acidic buffer of pH 1 (27). On the other hand,

antistreptococcal antibodies that were fixed on a column of immunoadsorbent–streptococcal polysaccharide–sepharose, were eluted by a gradient of decreasing pH and increasing saline concentration in the form of two successive fractions with different idiotypic specificities (28).

Therefore, it is extremely likely that, in our experiments, the antibodies to hen Ov that were eluted by different concentrations of $MgCl_2$ also possessed different immunochemical properties or functions, i.e., they were either specific to different antigenic determinants or possessed different affinities toward these determinants.

(*ii*) The existence of different immunochemical properties among antibodies that combine with the immunoadsorbent of the homologous antigen hen Ov and not of turkey Ov, or with turkey Ov but not with duck Ov, and finally among those antibodies that also combine with duck Ov, can hardly be doubted. It is, however, difficult to know if one is dealing with differences in antibody specificity or affinity, on the assumption that the distinction between these two properties is actually valid.

(*iii*) The difference between the immunochemical properties of the antibodies of fractions PA and NPA is equally clear, with the same reservations as to the precise nature of this difference. The difference between the antibodies of these two fractions and the proteins of fraction NRP is still more obvious since, not only do the latter proteins not combine with hen Ov, but they are even unable to agglutinate erythrocytes coated with hen Ov, nor elicit PCA with hen Ov. It might be speculated that the polymerization of hen Ov by glutaraldehyde might have made certain antigenic determinants disappear, and that fraction NRP might have contained antibodies against these determinants. The negative hemagglutination reaction does not provide conclusive evidence against this possibility, since glutaraldehyde was used to combine hen Ov with the erythrocytes. But this possibility would be much more difficult to reconcile with the absence of PCA, and with the fact that the fraction had also been passed through a column of another immunoadsorbent that was prepared by a different procedure without the use of glutaraldehyde. In any case, even if it were assumed that proteins of the NRP fraction that carry the same idiotypic patterns as those of the other fraction(s) might be endowed with antibody function against certain determinants that were absent from the hen Ov polymer, this assumption would imply that the same idiotypic pattern was present on antibodies with specificity for different determinants. It is probably more reasonable to assume that the idiotypes detected in the NRP fraction are proteins without antibody function. It is apparently the first time that idiotypic specificities have been observed in immunoglobulins without detectable antibody function, something that had not been foreseen when the definition was proposed.

Thus, it seems clear that the same idiotypic specificities may be found in antibodies to hen Ov with different functions and in immunoglobulins without detectable antibody function, which appeared in the serum after an immunization against hen Ov.

The hypothesis of an idiotypic specificity common to antibodies with different specificities had been discussed and considered likely in order to explain certain observations that were made with antibodies against *S. typhi* (8, 10). The three series of observations reported in this paper support this hypothesis in three precise cases, with the supplementary observation, which had not been foreseen, of idiotypic specificities common to antibodies and to proteins that have no antibody function. It had been considered likely that the specialization of the cells, which synthesize immunoglobulins, previously demonstrated (with certain reservations) from the triple standpoint of isotypic and allotypic specificities, and antibody functions of the synthesized immunoglobulins, would include also idiotypic specificities. It thus seems reasonable to conclude that immunoglobulins that differ from each other by one or the other of these four properties are synthesized by different cells. On the other hand, the fact that each idiotypic specificity is somewhat unique in the hen Ov and *S. typhi* systems [and even in the other idiotypic systems in which heterologous reactions were observed, the first of which was the *Salmonella abortus equi* system (6, 14, 15)], makes it reasonable to also suppose that when two immunoglobulin molecules of one given individual carry the same idiotypic pattern, this does not occur by mere randomness, but because of something common in the cellular origin of the immunoglobulins (8, 10). On the basis of these premises, as far as they are justified, one is led to attribute a common cellular origin to the different antibodies and proteins that do not possess antibody properties in which the same idiotypic specificities were found. This does not necessarily imply, of course, that, in contradiction to the cellular specialization mentioned above, these various immunoglobulins were synthetized by the same cells, but rather that the supposedly different cells that synthesized them would have had a common ancestral cell. The common idiotypic specificity of immunoglobulins, despite their different functions, would be, in a sense, its distinguishing feature. Thus, one would have to assume that the cells of a given line would have diversified by a process of differentiation not yet known, so that certain generations of cells that derived from them would synthesize proteins without antibody function and antibodies to hen Ov with different functions.

These observations supply a further opportunity for discussing the possible relationships, similarities, and differences, of what may be known of, and deduced from, the individual antigenic specificities of myeloma proteins (29, 30) and the idiotypic specificities of antibodies. It seems most unlikely that observations similar to the above may be made with myeloma proteins, and the same is also true for other observations on idiotypy of antibodies, as has been discussed elsewhere (9). For this reason, and in spite of what may be reasonably supposed to be common in the structural basis of these two kinds of antigenic specificities of molecules of pathological or physiological origin, it seems reasonable to conclude that the two phenomena should be considered separately.

The observation of the same idiotypic specificities on certain IgG and on certain IgM antibodies in the hen Ov system, as in the *S. typhi* system (10), emphasizes the general character of this observation.

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Correction. In the article "Photo-Affinity Labels for Adenosine 3′:5′-Cyclic Monophosphate", by D. J. Brunswick and Barry S. Cooperman, which appeared in the August 1971 issue of *Proc. Nat. Acad. Sci. USA*, **68**, 1801–1804, in the structure shown on p. 1801, the fourth (IV) derivative of cAMP had a subscript 2 omitted and should read: IV $R_1 = R_2 = -CO(CH_2)_2CH_3$.

Correction. In the article entitled "Organelle Mutations and Their Expression in *Chlamydomonas reinhardi*", by Stefan J. Surzycki and Nicholas W. Gillham, which appeared in the June 1971 issue of *Proc. Nat. Acad. Sci. USA*, **68**, 1301–1306, the following changes should be made. On p. 1303, Table 2, footnote *e*, should read: "Streptomycin-dependent". On p. 1304, left-hand column, beginning 14 lines from bottom, should read: "should exhibit a wild-type phenotype ...", not "should inhibit a wild-type phenotype ...". On p. 1305, Table 4, column head should read: "Location of mutation (Chlp./Mit. Nuc.)", not "Location of mutation (Chlp. Mit./Nuc.)".