LOCALIZATION OF ANTIBODIES IN GROUP I AND GROUP II γ-GLOBULINS*

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Studies from several laboratories have demonstrated that the pathological counterparts of immunoglobulins, *i.e.* the myeloma proteins, Bence Jones proteins, and Waldenström-type macroglobulins, each fall into two groups on the basis of mutually exclusive antigenic determinants present on these proteins (1–3). Furthermore, normal immunoglobulins fall into similar antigenic groups, based on the presence of the same antigenic determinants permitting the classification of the pathological immunoglobulins. In a previous study it was demonstrated that human γ_2-globulin consists of approximately 60 per cent of group I molecules and 30 per cent of group II molecules (4). Preliminary evidence (4) indicated that antibodies occurred in both groups. The present study was undertaken to investigate this question more extensively with particular emphasis on quantitative aspects. The data obtained demonstrate that various isolated antibodies contained the group I and group II antigenic determinants. Certain antibodies, however, consisted primarily of group I molecules, and others showed a predominance of group II molecules.

Materials and Methods

1. Antisera specific to group I immunoglobulins and specific to group II immunoglobulins were prepared as previously described (1), by immunizing rabbits with purified Bence Jones proteins of group I and group II respectively. These antisera failed to react with Bence Jones proteins of the opposite group. The antisera utilized in this study were Nos. 618 and 743 against group II and Nos. 646, 739, and 788 against group I proteins.

2. Quantitative precipitin curves with I-131–labeled proteins were performed as previously described (4). The amount of protein in the precipitate and the per cent of radioactivity of the antigen in the precipitate were plotted against increasing amount of antigen in order to determine the per cent of antigen precipitated in antibody excess zone.

3. Zone electrophoresis on starch medium was performed as previously described (5). Protein determinations were performed by the Folin-Ciocalteu method (6).

4. *Rh antibody analyses:* Rh-positive erythrocytes were washed three times with 0.15 M sodium chloride and then incubated at 37°C for 1 hour with an equal volume of 1:10 dilution of several sera containing γ_2 incomplete anti-Rh antibodies. Subsequently, the cells were

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washed three times with 0.15 M sodium chloride and diluted to a 4 per cent suspension. One drop of these sensitized cell suspensions was added to serial twofold dilutions of group I and group II antisera from which the heterophile antibodies had been absorbed.

5. *Isolation of antibodies:* Anti-Rh antibodies were supplied by Dr. Kochwa and Dr. Rosenfield of Mount Sinai Hospital. These were eluted from hemolyzed red cells by acid and were known to be essentially free of non-specific γ-globulin (7).

Anti-A, anti-B, and antidextran antibodies were isolated by acid elution of specific precipitates made at equivalence with the respective antigens. Acetate buffer at pH 3.8 was used, followed by ammonium sulfate precipitation. In addition, washed equivalence point precipitates were dissolved in antigen excess for many of the agar plate experiments. One antidextran antibody, which was utilized for quantitative assay, was supplied by Dr. Elvin Kabat. The antibody had been isolated from a specific precipitate with dextranase (8).

Antiteichoic acid antibodies were prepared from equivalence point precipitates from various sera which contained elevated amounts of these antibodies. The precipitates were dissolved in excess teichoic acid for agar plate analysis.

Semiquantitative analyses of the isolated antibodies were carried out on agar slides. Serial twofold dilutions of the antibody were placed in wells and allowed to diffuse against the same two standard group I and group II antisera in troughs. In most experiments the initial antibody concentration was approximately 1 mg/cc. For the experiments illustrated in Fig. 1 the initial antibody concentrations were as follows: anti-A Th, 1.8 mg/cc; anti-A 66, 2 mg/cc; antiteichoic acid Es, 0.6 mg/cc; antiteichoic acid Ga, 0.8 mg/cc.

RESULTS

Qualitative Demonstration of Group I and Group II Molecules in Incomplete Anti-Rh Antibodies.—Three different techniques were used to demonstrate group I and group II molecules among incomplete anti-Rh antibodies.

(*a*) In ordinary analyses of incomplete anti-Rh antibodies, the latter are permitted to combine with Rh-positive erythrocytes, and subsequently an antiserum to γ_2-globulin (containing antibodies to many antigenic determinants) is used to agglutinate the sensitized cells. It was reasoned that if group I antibody molecules combine with the Rh-positive erythrocytes, then these cells should be agglutinated with an antiserum to group I Bence Jones proteins; similarly, an antiserum to group II Bence Jones proteins should agglutinate the Rh-positive erythrocytes sensitized with group II antibody molecules. Such experiments were performed.

The potency of antisera to group I and group II Bence Jones proteins was initially assessed by their capacity to agglutinate tanned sheep erythrocytes, sensitized with Fr II γ-globulin. As shown at the bottom of Table I, antiserum 618 (against group II Bence Jones proteins) was slightly more potent than antiserum 646 (against group I Bence Jones proteins), particularly since Fr II contains approximately 60 per cent group I and 30 per cent group II molecules.

The results of the agglutination tests are recorded in Table I. By this method the presence of group I and group II incomplete anti-Rh antibodies was demonstrated in five different sera. Quantitation of these data was not possible, but it appeared that in these individuals group I incomplete antibodies were more abundant than group II antibodies.

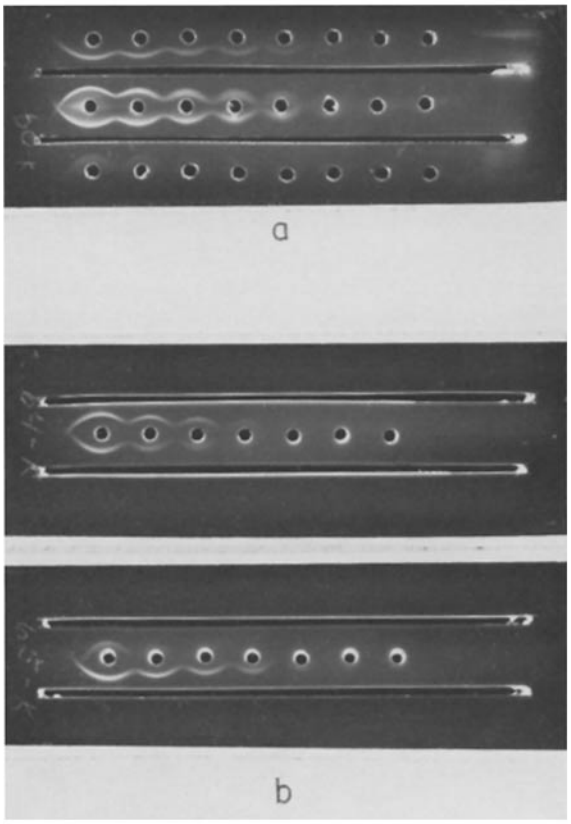

FIG. 1 Three agar slides showing the reaction of serial dilutions of isolated antibodies with group I and group II antisera. Upper trough in each slide contains antiserum 618 against group II; lower trough, antiserum 788 against group I.

(*a*) Isolated anti-A antibodies: Outer wells contain anti-A Th; inner wells anti-A 66. The anti-A Th reacts primarily with the group II antiserum, in contrast with the anti-A 66.

(*b*) Isolated antiteichoic acid antibodies. The wells in the upper slide contain antiteichoic acid Es which reacts slightly more with the group II antiserum. The wells in the lower slide contain antiteichoic acid Ga which reacts primarily with the group I antiserum.

(*b*) Physical means of separation of group I and group II molecules of γ_2-globulin have not been developed. Therefore, antisera to group I and group II Bence Jones proteins were used to precipitate one group of molecules as described previously (4). Accordingly, γ_2-globulin was isolated from two individuals with incomplete anti-Rh antibodies. With both isolated γ_2-globulins the zone of equivalence in precipitin curves was approximately 750 μg of protein per 1 ml of antiserum 646 (against group I Bence Jones protein) and for antiserum 618 (against group II Bence Jones protein). Therefore, to remain

in the antibody excess zone, 500 μg aliquots of both isolated proteins were precipitated with 1 ml aliquots of each antiserum. After precipitation with group II antiserum approximately 350 μg of the original protein remained in the supernatant, composed of group I molecules. Similarly, after precipitation with group I antiserum, approximately 200 μg of the original protein remained in the supernatant, composed of group II molecules. These supernatants were then tested for the presence of incomplete anti-Rh antibodies. After sensitiza-

TABLE I

Experiments Showing the Presence of Incomplete Anti-Rh Antibodies in Group I and Group II Molecules, Employing Group-Specific Antisera as Coomb's Sera

Serum used for sensitization of Rh cells	Antiserum used as Coomb's serum	Dilution of antiserum added to sensitized cells									
		1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Ga	646*	3+	3+	3+	3+	2+	2+	2+	1+	1+	0
	618‡	2+	2+	2+	2+	1+	1+	0	0	0	0
G1-2377	646	2+	2+	2+	2+	2+	2+	1+	0	0	0
	618	2+	2+	1+	0	0	0	0	0	0	0
62-508	646	2+	2+	2+	2+	2+	1+	1+	0	0	0
	618	2+	1+	1+	0	0	0	0	0	0	0
62-356	646	2+	2+	2+	2+	2+	2+	1+	1+	0	0
	618	2+	1+	1+	0	0	0	0	0	0	0
Ri	646	3+	3+	3+	2+	2+	2+	2+	1+	0	0
	618	2+	2+	1+	0	0	0	0	0	0	0
Saline for control	646	0	0	0	0	0	0	0	0	0	0
	618	0	0	0	0	0	0	0	0	0	0
Tanned sheep cells sensitized with Fr II	646	3+	3+	2+	1+	0	0	0	0	0	0
	618	3+	3+	2+	1+	1+	0	0	0	0	0

* 646 is an antiserum to group I determinants.

‡ 618 is an antiserum to group II determinants.

tion with 0.5 ml of each supernate and serial dilutions thereof, cells were washed and a commercial Coomb's serum was employed to test the presence of γ-globulin on the Rh-positive erythrocytes. The data of these experiments are recorded in Table II. It is clear that incomplete anti-Rh antibodies are present in group I and group II molecules.

(*c*) Small amounts of isolated anti-Rh antibodies from five individuals were analysed directly. Double diffusion experiments in agar were performed on serial dilutions of these preparations against antisera to group I and group II Bence Jones proteins. Exact quantitation of the amounts of group I and group II antibodies was not possible, but antibodies of both groups were present, and in three of five preparations group I antibodies predominated.

Qualitative Demonstration of Group I and Group II Thyroglobulin Antibodies.—

From six sera with high titers of thyroglobulin antibodies, γ_2-globulin was isolated by zone electrophoresis. With all isolated proteins the zone of equivalence in precipitin curves was approximately 750 μg of γ_2-globulin per ml of antiserum 739 (against group I Bence Jones protein) and antiserum 618 (against group II Bence Jones protein) as described above. 500 μg aliquots of each isolated γ_2-globulin were added to 1 ml of antiserum 618 and to 1 ml of antiserum 739 and the precipitates spun off. Serial twofold dilutions of 0.5 ml of the supernatants were carried out in 0.5 ml of 0.15 M sodium chloride solutions. To these dilutions were added 0.1 ml of thyroglobulin-sensitized erythrocytes.

TABLE II

Experiments Showing the Presence of Incomplete Rh Antibodies in Group I and Group II Molecules Separated by Precipitation with Specific Antisera

Antiserum 618 was used to precipitate group II molecules, 646 to precipitate group I molecules.

Sensitizing material	Concentration of γ_2-globulin	Type of molecules present	Dilution of sensitizing material and degree of agglutination observed							
			Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	$\mu g/ml$									
Isol. Ga γ_2	100	I + II	2+	2+	2+	1+	0	0	0	0
Isol. Ga γ_2 + 618	350	I	3+	3+	2+	2+	1+	0	0	0
Isol. Ga γ_2 + 646	200	II	3+	3+	2+	1+	0	0	0	0
Isol. Ri γ_2	100	I + II	3+	3+	2+	2+	1+	0	0	0
Isol. Ri γ_2 + 618	350	I	3+	3+	3+	3+	3+	2+	1+	0
Isol. Ri γ_2 + 646	200	II	2+	1+	1+	0	0	0	0	0
618	0		0	0	0	0	0	0	0	0
646	0		0	0	0	0	0	0	0	0

Agglutination was read after overnight incubation at 4°C, according to the pattern of settled erythrocytes. Control unsensitized cells were not agglutinated, and the antisera 739 and 618 alone caused no agglutination. In all instances both group I and group II molecules contained thyroglobulin antibodies.

Semi-Quantitative Analyses of a Variety of Isolated Antibodies.—Six isolated anti-A antibodies were studied with the group I and group II antisera in serial dilutions on agar slides. Fig. 1 *a* illustrates the results with two of these preparations. The majority of antibodies showed a distribution similar to Fr II with lines visible at higher dilutions with the group I antisera. However, there were two striking exceptions. Anti-A Th and anti-A Wh reacted much more strongly with the group II antisera. Anti-A Th is illustrated in the outer portion of Fig. 1 *a*. Similar results were obtained with antibodies isolated by acid extraction

or by solution in antigen excess. Analyses of these anti-A antibodies as to immunoglobulin composition with specific antisera for γ_2-, 19S, and β_{2A}-globulins demonstrated that anti-A Th and anti-A Wh consisted primarily of γ_2-globulin. Anti-A Wh also contained some β_{2A}-globulin. In density gradient experiments these two antibodies showed activity in the 7S and intermediate areas; there was no 19S activity. One isolated anti-A antibody was studied particularly because it contained a high concentration of 19S material. This showed strong reactivity with both the group I and group II antisera with a slightly higher reactivity with the group II antisera.

Two isolated anti-B antibodies were also studied. One of these was uniquely rich in β_{2A}-globulin. Both these antibodies showed approximately equal reactivity with the group I and group II antisera.

Three isolated antidextran antibodies were examined. Two reacted best with the group I antisera. The third reacted best with the group II antisera. These antibodies were studied simultaneously, and the difference in reactivity was striking. All three consisted of a vast predominance of γ_2-globulin.

Eight isolated antiteichoic acid antibodies were studied similarly (Fig. 1 *b*). Three showed a distribution similar to Fr II with a reaction with group I antisera at a slightly higher dilution than with group II antisera. Three showed approximately equal reactivity with the two types of antiserum. One showed a clearly increased reactivity with the group II antisera, and one showed a marked predominance of reactivity with the group I antisera. Antiteichoic acid antibodies were isolated from serum Th (used above for the anti-A system), and these were predominantly of the group I type. These antibodies were primarily of the γ_2 variety with some showing small amounts of β_{2A} and 19S immunoglobulins.

Quantitative Determination of Group I and Group II Molecules in Isolated Antibodies to A Substance and to Dextran.—Two of the isolated antibodies to A substance, which by the semiquantitative technique described above, contained more group II molecules than group I molecules, were prepared by acid extraction from specific precipitates and labeled with I-131. These preparations were used to construct quantitative precipitin curves with antisera to group I and group II Bence Jones proteins as previously described (4). The results of one of these experiments are illustrated in Fig. 2. The isolated γ_2-globulin from Th contained the normal distribution of 63 per cent group I molecules and 27 per cent group II molecules as shown in the antibody excess zone of the precipitin curve. On the other hand, the isolated anti-A antibodies from this serum consisted of approximately 30 per cent group I molecules and 60 per cent group II molecules. Approximately equal percentages of group I and group II molecules were present in the isolated anti-A antibodies from another individual.

One preparation of isolated antidextran antibodies, known to contain primarily group I molecules, was also studied by this quantitative technique. After I-131 labeling, these antibodies were found to consist of 92 per cent group I molecules and 8 per cent group II molecules.

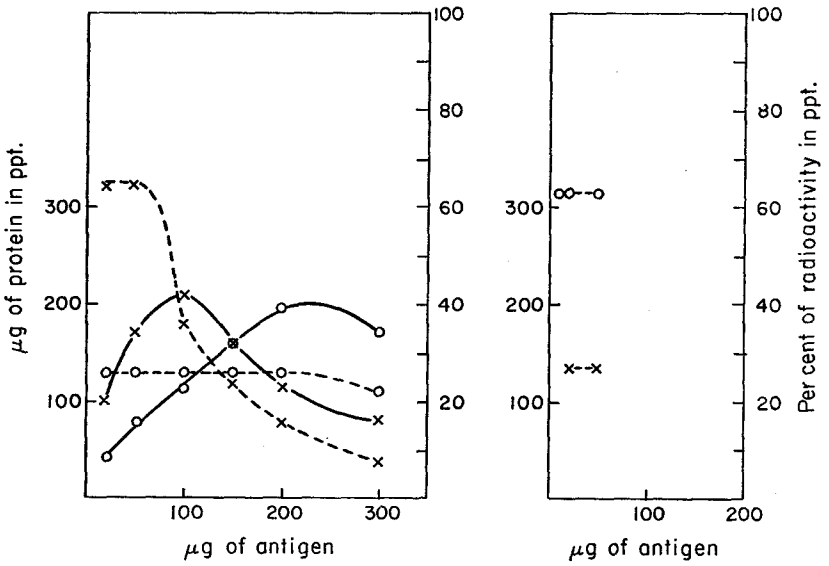

FIG. 2. Quantitative precipitin curves comparing the per cent of group I and group II molecules in γ_2-globulin and isolated anti-A antibodies from one individual. The left half of the figure illustrates γ_2-globulin isolated electrophoretically, labeled with I-131 and used to construct precipitin curves. x———x indicates protein and x - - - - x indicates per cent of radioactivity in the precipitate with antiserum 646 (anti-group I). o———o indicates protein, and o - - - - o indicates per cent of radioactivity in the precipitate with antiserum 618 (anti-group II). On the right-hand side similar data are given for the isolated anti-A antibodies from the same individual. x - - - - x indicates per cent of radioactivity precipitated by antiserum 646 (anti-group I), and o - - - - o indicates per cent of radioactivity precipitated by antiserum 618 (anti-group II).

DISCUSSION

The presented data indicate that both the group I and the group II molecules of γ_2-globulin carry antibody activity. All of the antibodies studied contained at least some molecules of each group. However, it is of interest that in certain instances antibodies were found in different ratios than the 60 per cent group I and 30 per cent group II molecules described previously for whole γ_2-globulin. This was clearly demonstrated for antidextran antibodies, where in one case quantitatively group I molecules predominated, while in another group II molecules were in excess. The excess of group II molecules was particularly

well documented in the case of two isolated anti-A antibodies. One of the anti-teichoic acid antibodies showed a great excess of group I molecules with only a slight reaction with the group II antiserum. All combinations were encountered ranging from a great excess of group II molecules to a great excess of group I molecules. It is of particular interest that two different antibodies, isolated from the same serum (Th), showed striking differences in the group I to group II ratio. In this work agar plate analyses proved particularly useful with antibodies dissolved in antigen excess; however, similar results were obtained when acid eluates were employed.

The exact reason for the variable ratio of group I and group II molecules in different antibodies is not clear at this time. Previous work with myeloma proteins and Waldenström macroglobulins demonstrated that these proteins, in at least the great majority of instances, belonged to one or the other group but never to both. In fact the incidence of γ_2-type myelomas of group I is approximately twice that of group II, which is very similar to the ratio of the two groups in whole γ-globulin (4). These proteins appear to be the products of single clones of cells, and perhaps the variable ratio of the groups in various isolated antibodies reflects the clonal distribution of the cells producing these antibodies. It seems particularly important to extend these observations on the ratio of groups to antibodies directed against a very limited number of antigenic determinants. Such antibodies appear to resemble the myeloma proteins in a number of respects, which is brought out particularly in the analyses of the L chains by gel electrophoresis (9). It is known that these chains carry the group determinants (10, 11). The possibility remains that antibodies directed against a single antigenic determinant might fall entirely into one or the other group.

Another possibility that requires consideration is that the group distribution among antibodies is related to the type of antigenic stimulus, or to the stage of immunization. No direct evidence on these points is available. The excess of group II molecules was most apparent with two anti-A antibodies, which might conceivably be related to this antigen. In addition, both of these antibodies were in considerable part of the intermediate sedimenting type (12), and this might be relevant. Evidence was obtained for both group I and group II antibodies in the other immunoglobulins (β_{2A} and 19S) as well, but the material was insufficient to permit quantitation of the ratios. It is interesting that β_{2A} and 19S antibodies to thyroglobulin have been found to possess both groups of antigenic determinants (13).

Recent studies of the amino acid composition and sequences of Bence Jones proteins and myeloma proteins as models of γ-globulins indicate that the group-specific antigenic determinants reflect marked differences in peptide composition (14, 15). It would appear on the basis of the wide assortment of antibodies studied that these structural differences exist within each type of

antibody. Since the ratios vary in different antibodies, observed differences in amino acid composition and peptide fingerprints between individual antibodies will have little meaning unless the role of the group variation is known.

A number of macroglobulins with biological activity found in certain human sera have also been studied. These include several cold agglutinins, one altered red cell agglutinin, and several unusual anti-γ-globulin factors. The question of whether these represent true antibodies has not been answered, and therefore these data were not included in the present study. In many ways they behave more like the classical Waldenström macroglobulins and appear monoclonal in type despite high levels in serum. Group analyses of these macroglobulins demonstrated that they were entirely of one group or the other and not both. This would also suggest that they are more closely allied to the pathological macroglobulins.

Two other types of analyses of isolated antibodies along with pathological proteins are currently under investigation in this laboratory. These include the individual antigenic specificity of antibodies (16) and alteration of the ratio of genetic factors (17). Evidence for a general correlation between these properties and unusual group distribution has been obtained. Anti-A Th which showed a striking excess of group II molecules was one of the best antibodies for demonstrating individual specificity. Other examples could be cited. In all these properties, along with the sharp electrophoretic banding of the L chains, antibodies to very limited determinants appear to approach the status of myeloma proteins.

SUMMARY

A wide assortment of antibodies has been analysed by qualitative and quantitative techniques for their content of group I- and group II-type γ-globulin molecules. These included Rh antibodies, isoagglutinins, thyroglobulin antibodies, dextran antibodies, and teichoic acid antibodies. All the antibodies studied showed the presence of both groups. However, the ratio of the two varied widely in different individuals and diverged markedly from the ratio in the total γ-globulin of the individual. Two antibodies isolated from the serum of the same individual showed different ratios for the two groups.

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