

BIOLOGIC ACTIVITIES OF AGGREGATED γ -GLOBULIN

VIII. Aggregated Immunoglobulins of Different Classes¹

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Previous studies demonstrated that nonspecifically aggregated rabbit and human γ -globulins, like antigen-antibody complexes, induce an increased permeability of guinea pig skin capillaries and fix guinea pig complement (C') (1-4). On a weight basis, the aggregated γ -globulin and antigen-rabbit antibody complexes were comparable with respect to the biologic activities. Ishizaka *et al.* (5) presented evidence that mechanisms of C' fixation by aggregated human γ -globulin were similar to those involved in C' fixation by antigen-antibody complexes. Rabbit albumin, α -globulin and β_1 -globulin developed neither skin reactive nor C'-fixing activities upon aggregation (6). Frommshagen and Fudenberg (4), who studied C'-fixing properties of macroglobulin from the sera of patients with Waldenstrom's macroglobulinemia and of myeloma proteins, found that the macroglobulin consisting of 19 S, 26 S and 45 S components did not fix C' either before or after heating at 63°C, and that similar heat treatment of some 7 S and 9.5 S γ A myeloma paraprotein failed to produce anticomplementary activity. Furthermore, the Fc fragment of normal rabbit γ G-globulin, obtained by papain digestion, induces both skin reactivity and C'-fixing activities upon aggregation, whereas F_{ab} fragment does not (7). These findings strongly suggest that structures in γ G-globulin molecules essential for the induction of these biologic activities are present in heavy chains.

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The work was presented before the 50th Annual Meeting of the American Association of Immunologists, which was held at Atlantic City, N. J., on April 14, 1966. This work was initiated when K. I. was at the Hematology Unit, University of California, San Francisco in 1965 as visiting Associate Professor.

Recent studies on γ -globulin structure have shown that structural differences among the three major classes of immunoglobulins, i.e., γ G, γ A and γ M, are restricted to their heavy chains rather than to their light chains (8-10). Antigenic subclasses of human γ G-globulin, i.e., γ_{2a} -, γ_{2b} -, γ_{2c} - and γ_{2d} -globulins, are also present in normal human serum (11-15). The distinguishing antigenic characteristics of each subclass are associated with the heavy chains (12). These findings suggested to us the possibility that biologic properties of aggregated γ -globulins differ depending on the classes of immunoglobulins and/or subclasses of γ G-globulin. The studies described below were undertaken to study this possibility.

MATERIALS AND METHODS

Human immunoglobulins. Normal human γ A-globulins were isolated from fresh serum by zinc precipitation method (16) with slight modifications (17). The normal γ M-globulin was obtained by the method described in a previous article (17). Multiple myeloma proteins were prepared by block electrophoresis on starch (18) or Pevikon C-870 (Fostabolage, Sweden) (19) from the sera of patients with multiple myeloma. A preparation of γ_{2d} -globulin was kindly supplied by Dr. W. D. Terry, National Cancer Institute. Samples of purified macroglobulin were obtained from the sera of patients with Waldenstrom's macroglobulinemia by gel filtration through Sephadex G 200 (Pharmacia, Uppsala, Sweden) columns followed by zone electrophoresis. One of the macroglobulin preparations (Bo) was cryoglobulin and another (Ev) was pyroglobulin.

Antisera specific for human immunoglobulins. The antisera specific for the γ G-, γ A- and γ M-globulins were the same preparations described in a previous article (17). In immunodiffusion (see below), each antibody preparation gave a single precipitin band of identical specificity with normal human serum and the respective immuno-

globulin but not with the other two immunoglobulins. A goat antiserum against whole human serum was also used.

The antisera specific for either kappa or lambda chains were prepared by immunizing rabbits with Bence-Jones proteins included in complete Freund's adjuvant (Difco Lab., Detroit, Michigan). Four milligrams of the protein were used for the initial immunization and 2 mg were given at 1-month intervals. Each antiserum was absorbed with Bence-Jones proteins of the opposite type. The antisera specific for subclasses of γ G myeloma proteins, e.g., γ_{2a} , γ_{2b} , γ_{2c} and γ_{2d} were prepared in rabbits by immunization with myeloma proteins (15). The myeloma proteins used as standards were previously typed with anti- γ_{2a} , anti- γ_{2b} , anti- γ_{2c} and anti- γ_{2d} monkey antisera by Dr. W. Terry, National Cancer Institute. The antiserum against each subclass of γ G-globulin was absorbed with a mixture of γ G myeloma proteins of the other three subclasses to render it specific for that subclass.

Complement and intermediate products. Fresh frozen guinea pig serum was purchased from Probio Inc., New York. Sheep erythrocytes in Alsever's solution were purchased from Colorado Serum Co., Denver, Colorado; the blood was obtained from the same sheep throughout the study. The guinea pig serum was absorbed twice with $\frac{1}{30}$ volume of packed sheep erythrocytes of 0°C and stored at -40°C. The first component of C' (C'1a) was purified from the guinea pig serum by the method described by Borsos *et al.* (20). The second component of C' (C'2) was kindly supplied by Dr. T. Borsos, National Cancer Institute. Rabbit antibody (A) to boiled stromas of sheep erythrocytes, sheep erythrocytes (E) sensitized optimally by A (EA) and the intermediate products consisting of EA and the first and fourth components of C' (EAC'1a,4) were prepared in the usual manner (21). The diluent for the reagents was Veronal buffer containing optimal concentrations of Ca^{++} and Mg^{++} and 0.1% gelatin (VB-saline) (21). The intermediate product consisting of EA and C'4 (EAC'4) was prepared by a modification of the method by Becker (22).

Aggregation of immunoglobulins. Immunoglobulins were aggregated by coupling with bis-diazotized benzidine (2). One hundred milligrams of benzidine dihydrochloride was dissolved in 10 ml of 0.25 N HCl and cooled to 0°C; then 2.0 ml of precooled 0.5 N NaNO_2 was added slowly with

stirring. After 30 min, the solution of bis-diazotized benzidine was diluted 10 times with cold distilled water. One-twentieth to $\frac{1}{4}$ ml of the diluted solution was added to 2 ml of 0.5 to 1% solution of immunoglobulins in borate buffered saline, and the pH was adjusted to 8.5. After 15 min to 1 hr, when the protein solutions became slightly opalescent, an equal volume of borate buffered saline was added and the mixtures were dialyzed against the same buffer for 24 hr. Insoluble aggregates were removed by centrifugation at 3000 rpm and the supernatant fluids containing biphenyl azo-proteins were used in subsequent experiments.

Ultracentrifugation. Analytical ultracentrifugation was carried out in a Spinco, model E Ultracentrifuge at $20 \pm 0.2^\circ\text{C}$ at 52,640 rpm. The relative apparent percentage of each component was determined from areas of the individual components. Corrections for the Johnson-Ogsten effect were not made.

Sucrose density gradient ultracentrifugation (23) was employed to separate aggregated γ M-globulin from nonaggregated protein. Four-tenths milliliter of the sample was applied to the top of the sucrose density gradient ranging from 20% on top to 50% at the bottom in a total volume of 4.2 ml. The tubes containing the gradient were placed in a swinging bucket rotor, SW 39, and centrifuged at 35,000 rpm for 16 hr.

Fractions of 0.5 ml were obtained from the top of the tubes and the pellets, if any, were resuspended in borate buffered saline.

Immunodiffusion. Double diffusion in agar plates was carried out by the method of Ouchterlony (24) and immunoelectrophoresis was performed following the modification of Scheidigger (25). The slides were incubated 2 days at room temperature and washed. The precipitin bands formed were stained with Amido schwartz 10B.

Classification of myeloma proteins. The classification and subclassification of myeloma proteins were carried out by immunodiffusion. On Ouchterlony plates, one of the antisera specific for γ_{2a} , γ_{2b} , γ_{2c} , γ_{2d} , γ A- and γ M-globulins was placed in a center well and reacted against the myeloma proteins in peripheral wells. Proteins of known antigenic subclass were used as a control. The γ G and γ A myeloma proteins and γ M-macroglobulin preparations were tested for the light chain type using antisera specific for κ and λ chains.

Gm typing of myeloma proteins. Gm factors were determined by standard inhibition of agglutination methods (26). Under the experimental condition, myeloma proteins positive for a given factor inhibited the agglutination in concentration of 0.01 mg/ml, whereas proteins negative for the factor failed to inhibit even at 2.5 mg/ml.

Complement fixation. The amount of C' fixed by immunoglobulin preparations was determined by the method of Mayer *et al.* (27). The samples were added to 100 C'H₅₀ (50% unit of C') in a final volume of 10 ml. After 19 hr at 3°C, the residual C' activity was determined. The concentration of each sample selected for testing was chosen so that the number of C'H₅₀ inactivated would represent 20 to 80% of the hemolytic activity present in the control containing only buffer and C'. From the number of C'H₅₀ fixed, the quantity of γ -globulin required to fix 50 out of 100 C'H₅₀ was calculated (28).

C'1 fixation tests were performed by a slight modification of the method described previously (29). The buffer used was VB-sucrose saline containing 0.001 M Ca⁺⁺ (30). The ionic strength of the buffer was 0.065. Appropriate dilutions of untreated and aggregated immunoglobulins were incubated with 0.5 ml of 1:40 dilution of guinea pig C' containing approximately 500 C'1H₅₀. A control tube contained only C'. The tubes were incubated for 20 min at 30°C with constant shaking and 4.5 ml of ice cold diluent were added to each tube followed by immediate centrifugation at 30,000 rpm for 30 min in the cold. C'1 activity in the supernatant fluids was measured using EAC'4, purified C'2 and guinea pig C' diluted with ethylenediaminetetraacetic acid (EDTA) as C'3 source (31); details of the procedure are described in a previous article (29). C'1a fixation tests were carried out using purified C'1a rather than whole guinea pig serum. Either 100 or 500 C'1aH₅₀ units were incubated with immunoglobulin and the C'1a activity left in the reaction mixture was measured without centrifugation.

Skin reactions. Tests for skin reactivity of immunoglobulin preparations were carried out in guinea pigs as previously described (1). Dilutions of the test preparations were injected into a shaved area of the back. The animals then received an intravenous injection of Evans blue and the reactions were measured from the inside surface of the skin 30 min later. The minimum

quantity of each preparation required to induce a reaction with average diameter of more than 8 mm was determined.

Reversed passive cutaneous anaphylaxis was carried out in guinea pigs by the method described by Ovary (32).

RESULTS

Normal immunoglobulin preparations and myeloma proteins were tested for purity by immunodiffusion using antisera specific for each of the three classes of immunoglobulins and anti-human serum. The preparations gave a single precipitin band of identical specificity with the anti-human serum and with the antiserum specific for the homologous immunoglobulins, but not with the antisera specific for the other two immunoglobulins. The sedimentation coefficients of γ A myeloma proteins were measured by analytical ultracentrifuge. Most of the preparations gave a single peak with a sedimentation coefficient (*s*_{20,w}) of 6.5 to 6.8 S. One purified γ A myeloma protein, composed of both polymer and monomer forms of which sedimentation coefficients were 6.4 S and 13 S, was applied to a Sephadex G 200 column to obtain polymer forms. Fractions corresponding to the first peaks were pooled and rechromatographed. The final preparation of the polymer form of γ A myeloma protein was mainly composed of a 13 S component.

The subclasses of γ G myeloma proteins, their content of the genetic factors and the results of light chain typing of γ G myeloma proteins are shown in Table I, and the data on γ A and γ M proteins are listed in Tables III and IV. The immunoglobulins were aggregated by coupling with bis-diazotized benzidine. The optimal dose of bis-diazotized benzidine required for aggregation differed for each class of immunoglobulins. For γ G-globulin, 50 to 100 μ g benzidine base/100 mg protein was sufficient, whereas two to three times as much was required for aggregation of γ A-globulins. Aggregated γ G- and γ A-globulins were obtained by centrifuging the biphenyl azo proteins at 100,000 $\times$ G for 4 hr in a Spinco preparative ultracentrifuge. The pellets and 0.5 ml of the solution at the bottom of the centrifuge tube were resuspended in borate buffered saline. The aggregated γ M-globulins were separated from nonaggregated protein by sucrose density gradient ultracentrifugation. After centrifugation of biphenyl azo- γ M-globulin at 35,000 rpm for 16

TABLE I
C'-fixing and skin reactivity of aggregated γ G-globulins

Subclass	Type	Subject	Gm			Inv		C'T ₅₀		Minimum Dose for Skin Reaction	
			a.	b.	f.	1	b	Untreated	Aggregated	Untreated	Aggregated
								μgN	μgN	μgN	μgN
γ 2a	K	Th	—	—	—	—	—	>800	45.7	>20	>16
	L	Wa	—	—	—	—	—	>800	35.0	>20	>20
γ 2b	K	Ba	—	—	+	—	—	119	8.2	>18	1.0
	K	To	—	—	+	—	—	>800	11.9	>16	2.0
	K	Sa	+	—	—	—	+	>800	30.9	>16	4.0
	K	Pa	—	—	+	—	+	>800	20	>16	1.0
	K	Bu	—	—	+	+	—	>800	33	>16	1.0
	L	Wr	+	—	—	—	—	>800	11.6	>16	1.0
	L	P	—	—	—	—	—	>800	9.0	>16	0.5
	L	Ga	+	—	—	—	—	307	16.2	>16	2.0
γ 2c	L	Vi ^a	—	—	—	—	—	>800	13.4	>16	1.0
	L	Br ^a	—	+	—	—	—	464	10.0	>16	2.0
γ 2d ^b	L	Me	—	—	—	—	—	>800	>800	>16	>8
	K	Du	—	—	—	—	—	>800	>800	>16	>8

^a Vi was positive for Gm (g), whereas Br was negative for the factor.

^b Aggregated γ 2d-globulins were purified by sucrose density gradient ultracentrifugation.

TABLE II
C'1 and C'1a fixation by aggregated γ G-globulins

Sub class	Type	Subject	C' H ₅₀ Fixed by 10 μgN ^a		C' 1aF ₅₀ ^b	
			Aggregated	Untreated	Aggregated	Untreated
					μgN	μgN
γ 2a	K	Th	355	0		
	L	Wa	357	35	3.1	>50
γ 2b	K	To	305	35	1.3	>50
	K	Sa	340	0		
	L	Pr	282	0	1.4	>50
	L	P	350	0	1.2	>50
	L	Ga	310	50	0.96	>50
γ 2c	L	Vi	415	0		
	I	Br	440	50	0.71	>50
γ 2d	L	Me			>50	>50
	K	Du			>50	>50

^a 500 C'1H₅₀ were added to the reaction system.

^b Amount required to fix 50 C'1aH₅₀ units out of 100.

hr in a swinging bucket rotor, fractions of 0.5 ml were collected and the pellets were resuspended in borate buffered saline. Each fraction was diluted in borate buffered saline, and absorption

at 280 m μ determined. A representative result is shown in Figure 1. When untreated γ M-globulin was applied to a control tube, most protein was detected in the fourth and fifth fractions and the pellet was not detectable. Thus, the pellet fraction from biphenyl azo- γ M-globulin was used as aggregated protein in subsequent studies.

The C'-fixing and skin reactive properties of untreated and aggregated γ G-globulins were studied. The dose of protein N required to fix 50 C'H₅₀ out of 100 is shown in Table I. It is evident that γ G myeloma proteins, except γ 2d-globulin, fix C' upon aggregation. Some of the untreated myeloma proteins were anticomplementary but the aggregated products showed much higher C'-fixing activity than the untreated protein. The untreated and aggregated proteins were injected intracutaneously into normal guinea pigs to test skin reactive properties. The aggregated γ 2b- and γ 2c-globulins gave definite skin reactions with 0.5 to 2 μgN , whereas as much as 16 to 20 μgN of the untreated protein did not. The aggregated γ 2a- and γ 2d-globulins did not increase the permeability of guinea pig skin capillaries (Table I). Since the extent of aggregation for γ 2d-globulin by coupling with bis-diazotized benzidine was less than that for other proteins,

TABLE III
C'1-fixing and skin reactivity of γA -globulins

Type	Subject	Inv(1)	Treatment	C'F ₅₀	C'1H ₅₀ Fixed by 50 μ gN ^a	C'1aF ₅₀ ^b	Skin Reactivity
				μ gN		μ gN	μ gN
K	Lo	+	Aggregated	212			>18
			Untreated	80			>20
L	Jo	—	Aggregated	340			>18
			Untreated	73			>20
L	Cl	—	Aggregated	>800	32	>50	>16
			Untreated	>800	0	>50	>20
L	Br	+	Aggregated	390	0	>50	>20
			Untreated	375	0	>50	>20
L	H	—	Untreated ^c	>800		>50	>20
K.L.	normal		Aggregated	>800	0	>50	>20
			Untreated	>800	0	>50	>20

^a 500 C'1H₅₀ were added to the reaction system.

^b Amount required to fix 50 C'1aH₅₀ out of 100.

^c Polymer form γA .

TABLE IV
C'1-fixing and skin reactivity of γM -globulins

Type	Subject	Inv(1)	Treatment	C'F ₅₀	C'1H ₅₀ Fixed by 30 μ gN ^a	C'1aH ₅₀ Fixed by 30 μ gN ^b	Skin Reactivity
				μ gN			μ gN
K	V	—	Aggregated	108	315		>9
			Untreated	>800	15		>16
K	C	—	Aggregated	63.8	140		>9
			Untreated	>800	0		>16
K	Bo ^c	+	Aggregated	115	346	180	>20
			Untreated	>800	0	0	>20
L	Ka	—	Aggregated	105	292	140	>9
			Untreated	>800	62	0	>16
L	E ^d	—	Aggregated	283	400	175	>20
			Untreated	700	0	0	>20
K.L.	Normal		Aggregated	110	385	145	>16
			Untreated	685	25	5	>16

^a 500 C'1H₅₀ units were added to the reaction system.

^b 500 C'1aH₅₀ units were added to the reaction system.

^c Cryoglobulin; C' fixation was carried out at 37°C.

^d Pyroglobulin.

aggregated protein was obtained by heating another preparation (Du) of γ_{2d} -globulin at 63°C for 20 min (1), followed by centrifugation at 100,000 $\times$ G for 4 hr. The pellet fraction showed

neither C' fixing nor skin reactive properties. In order to confirm the lack of the biologic activities, aggregated γ_{2d} -globulin was further purified by sucrose density gradient ultracentrifugation.

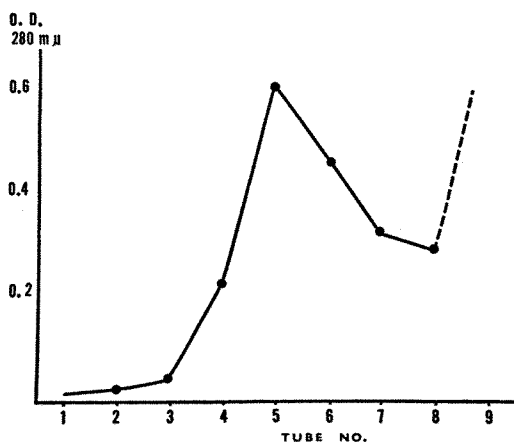


Figure 1. Density gradient ultracentrifugation of biphenyl azo- γ M-globulin (V). After centrifugation, 0.5 ml fractions were obtained from top. Fraction 9 contained pellet and turbid. Each fraction was diluted four times with saline and absorption at 280 $m\mu$ was measured.

Two-tenth milliliter of the pellet fractions of both heated and biphenyl azo γ_{2d} -globulins were layered on top of sucrose gradients from 12% on top to 37% at the bottom (23). The tubes were centrifuged at 35,000 rpm for 18 hr. After centrifugation, nine fractions were obtained from the top of the tubes. The pellets and 0.5 ml of the solution at the bottom of the tubes were resuspended in borate buffered saline and concentrated by negative pressure dialysis against the buffer. Nonaggregated protein was detected in the third to fifth fractions from the top. The bottom fractions from heated and biphenyl azo γ_{2d} -globulin fixed very little, if any, C' and gave no skin reaction in normal guinea pigs (Table 1). It is evident that the ability of the aggregated γ G-globulins to induce skin reactions depends on the subclass of the original protein. The results also showed that the light chain types and Gm factors studied had no relation to the induction of either C'-fixing properties or skin reactivity upon aggregation.

It has been reported by Terry (14) that γ_{2b} -, γ_{2c} - and γ_{2d} -globulins induce reversed passive cutaneous anaphylaxis in guinea pigs, indicating fixation of the proteins to animal skin tissues for passive sensitization. Lack of skin reaction by aggregated γ_{2d} -globulin suggested that the γ_{2d} -globulins used in the present experiments may not sensitize guinea pigs for reversed passive cutaneous anaphylactic (PCA) reactions. Reversed PCA reaction tests were therefore set up using

γ_{2a} -, γ_{2b} - and γ_{2d} -globulins. It was found that 0.1 to 0.2 μ gN of all myeloma proteins, except γ_{2a} -globulin, induced bluing reactions when guinea pigs were challenged with 100 μ gN of anti- γ G antibody.

Since the C'-fixation involves many components of C', C'1 fixation by the aggregated proteins was studied. The experiments were carried out in two ways. In one experiment, 500 C'1H₅₀ units were incubated with 10 μ gN of either aggregated or untreated protein and the C'1 activity left was measured. It was found that aggregated γ_{2a} -, γ_{2b} - and γ_{2c} -myeloma proteins fixed a significant amount of C'1, whereas nonaggregated protein did not. In another experiment, purified C'1a was used to study the fixation of C'1. When serial dilutions of aggregated γ G myeloma proteins were incubated with 100 C'1aH₅₀ units of purified C'1a for 30 min, C'1a units fixed as a function of the quantity of the aggregated γ -globulins followed a straight line in the range of 20 to 75% fixation (Fig. 2). In view of these results, the quantity of the protein required to fix 50 C'1aH₅₀ out of 100 was calculated for each preparation (28). The results of C'1a fixation were

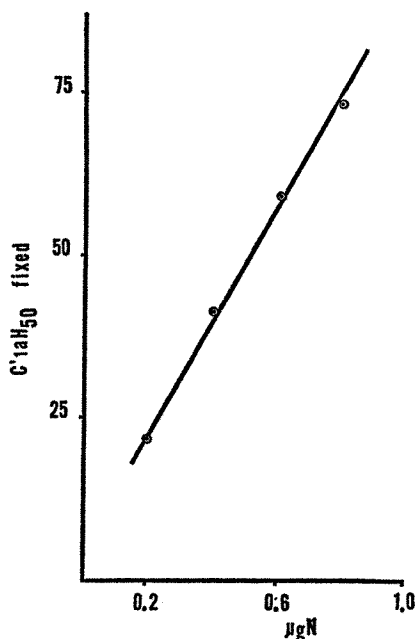


Figure 2. Relation between the dose of aggregated γ G-globulin (Br) added and C' 1aH₅₀ units fixed. The aggregated γ -globulin was incubated with 100 C' 1aH₅₀ units of purified C' 1a and the activity remaining was measured using EAC'4 (see text).

similar to those in C'1 fixation and confirmed that aggregated γ_{2a} , γ_{2b} and γ_{2c} myeloma proteins fixed C'1a whereas nonaggregated protein did not. Among the subclasses of γ G myeloma proteins, γ_{2d} -globulin did not induce C'1a-fixing ability upon aggregation (Table II).

The experiments were extended to γ A-globulins from both normal individuals and myeloma patients. Some of the γ A myeloma proteins were anticomplementary but no increase of C'-fixing activity by aggregation was observed in any of these cases. It is evident from Table III that aggregated γ A-globulin had neither C'-fixing activity nor skin reactive properties. Lack of C'-fixation by aggregated γ A-globulin was confirmed by C'1 and C'1a fixation tests. No significant amount of either C'1 or C'1a was inactivated by 50 μ gN of aggregated γ A-globulin. Furthermore, the polymer forms of γ A-globulin had neither C'-fixing properties nor skin reactivity.

The C'-fixing and skin reactivity of γ M preparations were also studied. Some of the untreated γ M-globulin were anti-complementary. By analytical ultracentrifuge, it was found that these preparations contained a small amount of soluble aggregates of which sedimentation coefficient ($s_{20,w}$) was 29 to 30 S. In order to remove the aggregates, the preparations were fractionated by sucrose density gradient ultracentrifugation. The concentration of sucrose gradient and the conditions for ultracentrifugation were the same as those for the separation of aggregated γ M-globulin. After centrifugation, 10 fractions were obtained and absorption at 280 m μ was determined. The γ M-globulin made a single peak at the middle of the sucrose gradient (cf. Frs. 4 to 6 in Figure 1) but the bottom fractions also contained some protein. Fractions 4 to 6 which corresponded to the major peak were pooled and concentrated by negative pressure dialysis. This procedure greatly reduced the anti-complementary activity of untreated γ M-globulin. The results of C' fixation by aggregated and nonaggregated γ M-globulins are shown in Table III; it is evident that aggregated γ M-globulin had significantly higher C'-fixing properties than nonaggregated proteins. In the case of γ M-cryoglobulin (Bo), however, a significant C'-fixation by nonaggregated protein was observed when the reaction was carried out at 2 to 3°C. Therefore the C' fixation reaction for both nonaggregated and aggregated proteins were performed at 37°C. The C' fixation by non-

aggregated protein was negligible, whereas the aggregated material fixed a significant amount of C' (Table III). The possibility arose that the increase of C' fixation upon aggregation might be due to coupling with benzidine itself rather than to aggregation. In order to study this possibility, nonaggregated biphenyl azo γ M protein was studied. After sucrose density gradient ultracentrifugation of biphenyl azo γ M-globulin (Ka), both pellet fraction and middle fractions (Frs. 4 to 6 in Fig. 1) were obtained and tested for C'-fixing properties. Even 800 μ gN of the middle fraction did not fix a significant amount of C'. The C'1 and C'1a fixation tests were also performed. The difference between the activities of aggregated and nonaggregated macroglobulins was clearly shown in these tests (Table IV). These results indicate that γ M-globulin induces C'1 fixing activity upon aggregation. However, the aggregated γ M-globulins did not increase permeability of guinea pig skin capillaries.

DISCUSSION

The C'-fixing properties of aggregated myeloma proteins were studied by Morse (33), but in her study, the myeloma proteins were not typed for subclass and quantitation of C'-fixation was not performed. The data presented in the present paper clearly show that the biologic activities of aggregated γ -globulins depend upon the class and subclasses of the original immunoglobulin. The aggregates of γ_{2a} -, γ_{2b} -, γ_{2c} - and γ M-globulins fixed C'1, whereas aggregated γ_{2d} - and γ A-globulins did not. Further aggregated γ_{2b} - and γ_{2c} -globulins increased permeability of guinea pig skin capillaries, whereas aggregated γ_{2a} -, γ_{2d} -, γ A- and γ M-globulins did not. The C' fixation by γ M-globulin aggregates apparently differ from previous reports that heat-aggregated γ M-globulin did not fix C' (4); Murray *et al.* (34) showed that γ M antibody lost C'-fixing activity after heating at 60°C. The lack of C' fixation by heat aggregated γ M-globulin is probably due to denaturation of the original molecules. The possibility has to be considered that the induction of C'1 fixing activity by the formation of biphenyl azo γ M-globulin might be due to the coupling of benzidine rather than to aggregation. However, the 19 S fraction of the biphenyl azo protein did not show any C' fixing activities. The present results also suggested that the induction of C' fixing activity by aggregation is not restricted to

this method. Removal of naturally aggregated γ M-globulin from 19 S fraction by density gradient ultracentrifugation resulted in a decrease of anti-C' activity of the γ M-globulin preparations. It was also found that γ M-cryoglobulin fixed C' at 2°C but not at 37°C. Presumably the cryoglobulin formed aggregates at low temperature and fixed C'.

The C'1 and C'-fixing activities of the aggregated γ M-globulin were much less on a weight basis than those of the aggregated γ G-globulins. The reasons for the quantitative difference must await further studies; however, it might be due mainly to technical difficulties of preparing γ M aggregates of small size. With the aggregated γ G-globulins, it was found that insoluble aggregates had less C' fixing activity than soluble aggregates. More recent studies indicate that soluble rabbit γ M aggregates have had very high C'-fixing properties (35).

The present data show a good correlation between antigen-antibody complexes and non-specifically aggregated γ -globulins with respect to biologic activities. It has been reported that both γ G and γ M antibodies fixed C'1 and C'1a with antigen, whereas γ A antibody did not (29). In regard to an increased permeability of guinea pig skin capillaries, Terry (14) reported that human γ_{2b} -, γ_{2c} - and γ_{2d} -globulins induced reversed PCA reactions in guinea pigs, whereas γ_{2a} -globulin did not. The results indicated that γ_{2a} -globulin molecules lack affinity for guinea pig tissues for passive sensitization. We confirmed the results with the materials used in the present experiments. It also has been reported that neither γ A nor γ M antibodies (globulins) induced either direct or reversed PCA (17, 36, 37). The present data on skin reactivity of the aggregated γ -globulins agree with previous reports and support the idea that combination of aggregated γ -globulin with certain tissue sites to which antibody is fixed upon sensitization is one of the necessary prerequisites for the induction of skin reactions. However, fixation of aggregated γ -globulin to the tissues is not sufficient for the induction of the skin reactions. γ_{2d} -Globulin fixes to the tissues but aggregates of the protein did not induce the skin reaction. Our more recent studies on soluble antigen-antibody complexes composed of guinea pig γ_1 antibody indicate that the complexes lacking C'-fixing properties induce skin reactions in guinea pigs. It is possible that

the increased permeability of guinea pig skin capillaries may be induced by two different mechanisms, one of which involves C' fixation and another which does not. Another possibility is that some unknown structures in γ -globulin (antibody) molecules, as well as those involved in the fixation to the tissues, are necessary for the formation of skin reactive aggregates. Further studies are necessary to elucidate this point.

With respect to C'1a fixation by γ M antibody, Borsos and Rapp (38) reported that a single molecule of γ M antibody on red cell surface generates a site for C'1 fixation. Their observation was confirmed in our previous experiments, using type A human erythrocytes and γ M isoagglutinin (29). However, our more recent studies on C' fixation by complexes of A substance and γ M anti-A antibody showed a definite inhibition of C' fixation in antigen excess region. The findings with the soluble antigen-antibody system suggest that aggregated γ M, but not a single molecule of γ M-globulin, fixed C'. Explanations for the difference between particulate antigen systems and soluble antigen systems must await further studies. As suggested previously (2), it is possible that the induction of C'-fixing activity by antigen-antibody reactions is due to distortion of antibody molecules. In the case of γ M antibody which has five to six combining sites in a molecule (39), the antibody may combine with a red cell surface through multiple sites and consequently induce distortion of the molecule which may also be induced by non-specific aggregation. Studies are currently in progress using rabbit anti-A γ M antibody which may aid in clarification of this point.

The present data clearly show that the skin reactivity and C'-fixing activities of aggregated γ -globulins are related to heavy chain structures rather than light chains. None of the Gm nor Inv factors studied appear directly related to the induction of the biologic activities. These findings are in agreement with the evidence that the structures essential for C' fixation and skin fixation are present in heavy chains in the immunoglobulin molecules (7). The relation between the structures involved in one of these activities have been studied in rabbit γ G-globulin (40) and guinea pig antibodies. Lack of C' fixation by guinea pig γ_1 antibody, which sensitizes homologous skin, indicates a difference between the structures (41, 42). The present data support

and extend these findings through the demonstration that aggregated γ_{2a} -globulin fixed C'1 but induced no skin reaction whereas γ_{2d} -globulin fixing to skin tissues did not fix C'1 upon aggregation. These findings are in accord with results of peptide map studies showing that Fc fragment of γ_{2d} -globulins differs markedly from the comparable fragment of γ_{2a} , γ_{2b} and γ_{2c} proteins, though these three are very similar to each other (43). It is apparent that the part of γ -chain structure which is essential for C' fixation is different from those involved in skin fixation.

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SUMMARY

Human myeloma proteins, γ_{2a} , γ_{2b} , γ_{2c} , γ_{2d} and γA , normal γA -globulin and γM -globulin from normal individuals and from Waldenstrom macroglobulinemia patients were aggregated by coupling with bis-diazotized benzidine. The aggregates and the untreated proteins were studied for complement-fixing properties and skin reactivity in normal guinea pigs. Neither untreated nor aggregated γ_{2d} - and γA -globulins showed either activity. The other immunoglobulins, e.g., γ_{2a} , γ_{2b} , γ_{2d} and γM , induced C' fixing properties upon aggregation. The results were confirmed by C'1 fixation tests. The aggregated γ_{2b} - and γ_{2c} -globulins induced increased permeability of guinea pig skin capillaries, whereas neither aggregated γ_{2a} - nor γM -globulin did. These activities of aggregated γ -globulins were independent of types of light chains and of the presence of Gm factors (a) (b) and (f). The results indicate that the heavy chain structures which are essential for C'1 fixation are different from those involved in fixation of the γ -globulin molecules to guinea pig tissues for passive sensitization.

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