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Biological Activities of Aggregated Gamma Globulin I. Skin Reactive and Complement-Fixing Properties of Heat Denatured Gamma Globulin.* (25116)

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In earlier publications on skin reactivity and complement (C′) fixing potency of soluble antigen-antibody complexes, it was shown that these biological activities are dependent on 2 properties of the complexes, *i.e.*, their composition with respect to antigen/antibody ratio and the species from which the antibody was derived(1,2). When complexes were composed of immune reactants in the molar ratio Ag_2Ab or when the antigen was a simple hapten, the biological activities of skin reactivity and C′-fixation were lacking. Moreover, it was observed that formation of soluble complexes with biological activities was accompanied by increase in levorotation (3). A mechanism for the observed changes in optical rotation was suggested, which postulated that the increase in levorotation might be due to interaction between antibody molecules brought into apposition by antigen. The possibility was also considered that this antibody-antibody interaction might result in induction of skin reactivity and fixation of C′ (3). These considerations were restricted to those complexes containing human or rabbit antibody which manifested the biological activities. It was therefore anticipated that the interaction of human or rabbit antibody (gamma globulin) molecules, induced by reagents other than specific antigen, might also be accompanied by a development of skin reactive and C′-fixing properties. Since it has been shown that the molecular configuration of gamma globulin changes on heating with formation of aggregates, this denatured protein was used to test this possibility. The experiments described below indicate that human gamma globulin aggregated by heat can provoke skin reactions in normal guinea pigs and can also inactivate C′. Aggregated bovine gamma globulin exhibits neither of these properties.

Materials and methods. Gamma globulin. Two preparations of human gamma globulin (HGG) were used, a pooled commercial human Fr. II (Squibb) and a preparation obtained through the courtesy of Dr. W. L. Hughes.‡ The bovine gamma globulin (BGG) was Fr. II (Armour & Co.). In free boundary electrophoresis in barbital buffer (pH 8.6, $\mu = 0.1$), the purified HGG (Hughes) showed a single sharp peak. The BGG contained approximately 5% of a component with mobility of -2.6×10^{-5} cm^2 volt^{-1} sec^{-1}. The tests for skin reactivity of gamma globulin were carried out in guinea pigs in the same manner previously described for testing activity of soluble antigen-antibody complexes(4). The gamma globulin preparations were injected in

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volumes of 0.1 ml into the shaved skin of the back. The animals then received an intravenous injection of Evans Blue and the reactions were estimated from inside of skin 30 minutes later. *Complement fixation test.* Pooled guinea pig serum was used as the source of C′. It was obtained in frozen state from Hyland Laboratories, Los Angeles, Calif. The amount of C′ inactivated by gamma globulin preparations was determined by the method of Mayer *et al.*(5). The diluted test samples were added to 100 $C'H_{50}$ (50% units of C′) in final volume of 10 ml. After 19 hours at 4°C, the residual C′ activity was determined. The number of $C'H_{50}$ inactivated was calculated by subtraction of units left after incubation from the number present in the control containing only buffer and C′. Concentration of each sample for testing was selected so that the number of $C'H_{50}$ inactivated would represent 20 to 80% of hemolytic activity present in the control. $C'F_{50}$, the quantity of each preparation required to fix 50 out of 100 $C'H_{50}$, was calculated according to the method described in(6). C'_3 titrations were carried out by the method of Rapp *et al.* (7). Guinea pig serum was treated with formalin and then reacted with sensitized sheep erythrocytes to yield the intermediate reaction product, $EAC'_{1,4,2}$. These cells served as substrate for estimation of C'_3 in terms of C'_3H_{50}. The quantity of gamma globulin required to inactivate 50 C'_3H_{50} out of 100 units was calculated as described above for determination of $C'F_{50}$.

Results. A. *Skin reactive properties.* The first studies were made with HGG. Aliquots of a 1% solution of HGG (Hughes) in borate buffered saline (pH 8.0) were heated 20 minutes at several temperatures ranging 56°C to 68°C. At temperature higher than 60°C, the preparations were opalescent and at 68°C a precipitate formed. Turbidities of preparations determined at 400 mμ in the Beckman spectrophotometer, model DU, are shown in Table I. Twofold dilutions of these preparations and of unheated HGG were tested for their capacity to enhance capillary permeability with results shown in Table I. It will be seen that HGG heated at 63°C showed augmented skin reactivity at a level of 3 μg N in a volume of 0.1 ml, whereas the unheated HGG did not show this property at a level of 32 μg N.

An attempt was made to purify the skin reactive component in the heated HGG preparation. Two g of Fr. II (Squibb) were dissolved in 100 ml borate buffered saline (pH 8) and centrifuged to remove the insoluble residue. The supernatant was precipitated with sodium sulfate at pH 8. The precipitates obtained at .62 mole/l of sodium sulfate and at 0.62 to 1.18 moles/l were designated Fractions A and B respectively. Both precipitates were dissolved in, and dialyzed against borate buffered saline. A moderate degree of opalescence was observed in Fraction A. Fraction B was entirely clear and showed a single peak ($\mu = -1.2 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}) in free boundary

TABLE I. Effect of Heating on the Skin Reactivity and Complement-Fixing Properties of Human Gamma Globulin.

Temp., 20 min., °C	Optical density*	Min skin reactive dose†	Quantity required to inactivate 50 $C'H_{50}$	
			In buffer‡	In EDTA (0.01 M)
		μg N		
Unheated	.019	>32	890	>>800§
56	.025	16	450	
60	.075	6	28	>800‖
63	.488	3	17	>800‖
68	4.281	>32	470	

* Optical density of solutions containing 1 mg N/ml at 400 mμ with 1 cm light path.

† These values indicate the minimum quantity required to induce a definite skin blueing, avg diameter more than 8 mm.

‡ The term buffer refers to isotonic veronal buffer containing levels of Ca^{++} and Mg^{++} optimal for hemolytic C′ activity(5).

§ No. of $C'H_{50}$ fixed with 800 μg N was negligible as compared with experimental error.

‖ No. of $C'H_{50}$ fixed with 800 μg N were 12 and 15 $C'H_{50}$ respectively.

TABLE II. Skin Reactivity and Complement-Fixing Properties of HGG and of Heated HGG Fractions.*

Treatment	Fraction	Sodium sulfate, mole/l	Optical density	Min skin reactive dose, μg N	Quantity required to inactivate 50 $C'H_{50}$		Quantity required to inactivate 50 C'_3H_{50}†	
					In buffer	In EDTA	37°C	0°C
					μg N			
Unheated	A	.62	.072	4.0	23	1000	400	>>800
	B	1.18	.019	64.0	1250	>>800§	>>800§	"
Heated	B_h		.358	2.0	6.9	1200	87	"
	Fr. 1	.36	.874	.5	2.8	830	39	"
	2	.62	.256	1.0	4.3	1000	63	"
	3	.81	.024	32	1140			
	4	1.18	.028	32	>800‡			

* See legend for Table I for description of terms.

† These values were obtained using same $EAC'_{1,4,2}$ cells at same time. No. of C'_3H_{50} units left in control tube was approximately 100.

‡ 800 μg N inactivated 13 $C'H_{50}$.

§ Inactivation of C′ or C'_3 activity with 800 μg N was negligible as compared with experimental error.

electrophoresis in barbital buffer of pH 8.6 and ionic strength of 0.1. The protein concentration of this fraction was adjusted to 2% and heated at 63°C for 20 minutes at which time the solution became markedly opalescent. This heated HGG (Fr. B_h) was fractionated again with sodium sulfate according to method described by Christian(8) to yield 4 different fractions. These were designated Fr. 1, Fr. 2, Fr. 3, and Fr. 4, and had been precipitated successively at 0.36 mole/l, 0.36 to 0.62 mole/l, 0.62 to 0.81 mole/l, and 0.81 to 1.18 moles/l of sodium sulfate. As indicated by optical density measurements in Table II, only the first 2 fractions of the heated HGG were opalescent. The data in Table II also show that Fraction A had skin reactive properties, but Fraction B did not. The increase of skin reactivity resulting from heating was also confirmed. The denatured protein in Fr. B_h, presumably responsible for its skin reactivity, was concentrated in Fr. 1 and 2 on precipitation with sodium sulfate. It may be noted that as little as 0.5 to 1.0 μg N of these fractions gave definite skin reactions.

Since bovine antisera generally lack sensitizing activities for guinea pigs(9), it was of interest to study the skin reactive properties of heated BGG. Aliquots of a 1% solution of BGG, at pH 8.0, were heated 20 minutes at 56°C, 63°C, and 65°C. The solution heated at 56°C was clear whereas the other 2 were opalescent with a turbidity comparable to analogous HGG preparation. These BGG preparations, however, failed to produce skin reactions with as much as 32 μ N.

B. *Complement-fixing properties.* Inactivation of C′ by HGG preparations described in the previous section was studied. Appropriate dilutions of the preparations were added to 100 $C'H_{50}$ units of C′ and incubated at 4°C for 19 hours. The C′-fixing potencies were compared in terms of $C'F_{50}$, *i.e.*, the quantity (μg N) of each sample required to fix 50 out of 100 $C'H_{50}$. The results obtained are shown in Tables I and II. It may be seen that the HGG heated at 63°C was much more active than the unheated control both with respect to C′ inactivation and skin permeability enhancement. The parallelism between skin reactivity and C′-fixing potency was also confirmed with the several fractions of heated HGG (Table II). It is perhaps worthy of special note that Fractions 1 and 2 were highly potent in C′-fixation and also showed highest skin reactivity.

In an attempt to compare the mechanism of C′ inactivation by heated HGG and by antigen-antibody complexes, the effect of a chelating agent was explored. It has been amply demonstrated that EDTA deprives the reaction system of the divalent cations required for interaction of C'_1, C'_4, and C'_2 with antigen-antibody complexes(10). In the present experiments, Na_3HEDTA, in final concentration of 0.01 *M*, was added to the reaction mix-

tures containing 100 $C'H_{50}$ and 1 ml of 0.5% HGG preparations in total volume of 4 ml. After incubation at 4°C for 19 hours, 0.4 ml of 0.1 *M* $CaCl_2$ was added to each mixture which was then diluted 50 times with barbital buffer, containing optimal concentrations of Ca^{++} and Mg^{++}, and the residual C′ was titrated. The data in Tables I and II indicate that inactivation of C′ by heated HGG is inhibited in presence of EDTA. It has been shown that inactivation of C'_3 by antigen-antibody reactions is dependent on temperature of incubation(11). These findings were duplicated in experiments with heated HGG. One ml volumes of appropriate dilutions of unheated HGG, of heated HGG, and of the several fractions were mixed with 100 $C'H_{50}$ of C′ in total volume of 10 ml. After incubation for 1 hour at 0°C or 37°C, the residual C'_3 activity in each reaction mixture was titrated with results shown in Table II. There was significant inactivation of C'_3 by Fraction A of unheated HGG, heated HGG (Fr. B_h) and its Fractions 1 and 2, in contrast to Fraction B of unheated HGG. It was also observed that inactivation of C'_3 is markedly dependent on temperature. At 0°C, no significant loss of C'_3 was observed with any of the preparations. The behaviour of heated HGG and of antigen-antibody complexes is therefore similar in this regard.

The C′-fixing potency of BGG preparations was also studied. One ml volumes of 0.5% solutions of either unheated or heated BGG (20 minutes at 56°C, 63°C, and 65°C) were incubated with C′ and the residual C′ was titrated. The anticomplementary effect of BGG was extremely low and not increased by heating.

Discussion. The present experiments clearly show that heat denatured preparations of HGG can enhance permeability of minute vessels in the guinea pig skin and fix C′. On fractionation of heated HGG with Na_2SO_4, these activities are concentrated in Fr. 1 and 2 (Table II) which are opalescent. The proteins in these fractions seem to be aggregated as judged in terms of decreased solubility and increased turbidity. With respect to unheated HGG, Fraction A obtained by precipitation with sodium sulfate (0.62 mole/l) also possesses both the biological activities under study. These experiments and those of Christian(5) indicate that salt fractionation provides a method for concentrating the aggregated gamma globulins which can induce the immediate type of skin reaction. It may also be noted that aggregated HGG (Fr. 1) yielded a definite skin reaction at a level of 0.5 μg N. This value is comparable to the minimum skin reactive dose of soluble antigen-antibody complexes. It was found previously(2) that the approximate amount of antibody in the minimum skin reactive dose of active complexes was 0.2 to 0.8 μg N. It is therefore evident that aggregated HGG and soluble antigen-antibody complexes are quantitatively comparable with respect to their skin reactive properties.

The data in Tables I and II show that skin reactivity and C′-fixing properties of heated HGG parallel each other, in agreement with the parallelism observed in studies with soluble complexes(2). The lack of skin reactivity shown by heated BGG is in accord with observations that activity of soluble complexes depends on properties of the antibody(2) and that bovine antibody is incapable of sensitizing guinea pigs(9). If the aggregated gamma globulin and the soluble complexes operate through similar mechanism, it would follow that the fixation of the altered gamma globulin to the tissues should be one of the necessary pre-requisites for the skin reaction(12). In this sense, inactivity of aggregated BGG might be due to low affinity for guinea pig tissues as well as its deficiency in C′-fixing capacity (*cf.* 12).

With respect to possible relationship of the present findings to C′-fixation, Davis, *et al.* (13) reported that HGG was anticomplementary and Olhagen(14) showed that aggregation of these proteins was responsible for this effect. In our study it was found that 2.8 μg N of Fraction 1 of heated HGG inactivated 50 out of 100 $C'H_{50}$. This value is of the same order of magnitude as that reported by Wallace, *et al.*(6) who found that the weight of rabbit anti-BSA required to fix 50 units out of 100 units in the cold with optimal amount of antigen varied from 1 to 3.94 μg N. Osler(15) has recently presented data for

human antibody showing that 2.36 μg N of human Wassermann antibody fixed 35 to 38 out of 50 $C'H_{50}$ of C′ with optimal amounts of cardiolipin antigen, although incubation time was only 90 minutes at 37°C in this experiment. From these comparisons it seems that the aggregated HGG and antigen-antibody complexes have C′-fixing potencies which are of the same order of magnitude. Inactivation of C′ by aggregated HGG requires divalent cation(s) as is also the case in fixation of C′ by antigen-antibody reactions(15). Inactivation of C'_3 and its dependence on temperature are also in agreement with the findings for C′-fixation by antigen-antibody complexes(10,11). The present results, therefore, suggest that the mechanism of C′ inactivation by heated HGG and by antigen-antibody complexes might be similar. Lack of C′-fixing properties shown by heated BGG is in agreement with the fact that only a small proportion of cattle develop C′-fixing antibody (16,17).

An explanation of the mechanism involved in skin reactions and C′ inactivation by aggregated HGG must await further studies of the aggregated protein as obtained by methods other than heat denaturation. However, comparable capacities of aggregated HGG and of antigen-antibody complexes with respect to skin reactive and C′-fixing properties suggest a similarity in mechanism. In our previous studies, evidence was presented that certain soluble antigen-antibody complexes show changes in molecular configuration of antibody and also exhibit skin reactive properties (3). It was suggested that this molecular change might be due to interaction between antibody molecules, brought into close proximity by antigen(3). The present experiments indicate that aggregated HGG, without participation of any antigen, can produce a toxic effect comparable to soluble complexes. This finding supports our hypothesis that the skin reactivity shown by the soluble complexes is due to antibody-antibody interaction and/or the consequent change in their configuration. In terms of this hypothesis, skin reactions might be produced as a result of combination of such altered antibody molecules to the tissues(3,12).

With respect to the mechanism of C′-fixation by antigen-antibody reactions, Heidelberger, *et al.*(18) postulated that components of C′ enter into easily dissociable compounds with antibody molecules in the absence of antigen and when antigen is introduced, the molecules of C′ become firmly bound in immune aggregates. Hill and Osler (19), in support of this hypothesis, concluded that C′-fixation increases with aggregating capacity of antibody. The present data may be considered to extend Heidelberger's idea through the demonstration that aggregated HGG by itself—without participation of any antigen—is entirely comparable to antigen-antibody complexes in inactivation of C′. If the mechanism of C′ inactivation by aggregated HGG is actually the same as the inactivation by antigen-antibody complexes, a possible mechanism of C′-fixation may be suggested. Thus, interaction between antibody molecules brought into apposition by antigen, with resulting change in their configuration, might be responsible for fixation of C′ by antigen-antibody reactions. This idea is in accord with the fact that neither the Ag_2Ab complex nor the simple hapten-antibody complex fix C′(2). Since aggregated BGG does not fix C′, the interaction between gamma globulin (antibody) molecules is probably not the only determinant of C′ fixation. The dependence of C′-fixing properties of antigen-antibody complexes on the species from which the antibody is derived suggests that the properties of antibody molecule may be another important determinant of C′ fixation. The hypothesis outlined above would therefore be restricted to those complexes containing antibody of the rabbit or human type.

Finally, our findings are also in accord with the hypothesis that C′ may be an essential reagent in the mediation of allergic reactions of the immediate type(11,20). The present data merely provide another set of correlated observations in this regard and suggest that a similar change of gamma globulin (antibody) might be responsible for both the skin reactivity and C′-fixing properties. In any event, these and previous observations(3) suggest that the interaction between gamma globulin (antibody) molecules might play an impor-

tant role in many biological phenomena which follow antigen-antibody combination, such as C′-fixation, skin reaction with soluble complexes, as well as anaphylactic reactions. It is of interest that aggregated gamma globulins give allergic-like responses and this suggests the possibility that some allergic dieases might be induced by molecular changes of the gamma globulin *in vivo*.

Summary. 1. Aggregated human gamma globulin, obtained by heating, gave immediate skin reactions in normal guinea pigs and inactivated complement, whereas aggregated bovine gamma globulin did not have either activity. 2. Aggregated human gamma globulin and antigen-antibody complexes are comparable on weight basis with respect to skin reactivity and complement inactivation. 3. Divalent cation(s) are required for inactivation of complement by aggregated HGG. Inactivation of C′3 by aggregated HGG is markedly dependent upon temperature. 4. A tentative hypothesis concerning the mechanism of complement fixation by antigen-antibody reaction is presented.

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Effect of Sodium Chloride Feeding on Adrenocortical Hormone Secretion of Salt Deprived Rats.* (25117)

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Sodium deprivation in rats results in increased secretion of aldosterone but total adrenocortical hormone production is decreased because of diminished synthesis of corticosterone and other steroids(1). These changes in hormone secretion take place gradually after withdrawal of sodium from the diet and require several weeks to become fully manifest. Alterations in adrenal steroid secretion induced by sodium restriction are reversible but it is not known how long it takes for cortical hormone production to become normal once adequate sodium intake is resumed. The purpose of this investigation was,

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