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Haemorrhagic Reactions Elicited at Sites of Passive Cutaneous Anaphylaxis by the Intravenous Injection of Aggregated γ-Globulin

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Summary. In guinea-pigs injected intradermally with a small amount of antibody and challenged 2 hours later, by the intravenous route, with a mixture of homologous antigen and aggregated γ-globulin, haemorrhagic reactions of the Arthus type develop at the sites of intradermal sensitization. This effect was obtained with γ-globulins of different species (human, rabbit and horse) by using different techniques for aggregation (heat, mercaptoethanol–urea and bis-diazobenzidine) and was always correlated with the ability of the aggregated globulin to fix complement.

Fluorescein labelled aggregates of γ-globulin were detectable in the wall of vessels at sensitized sites.

In experiments performed with guinea-pig antibodies, the localizing effect was observed only with γ_1, whereas the γ_2, Arthus-producing fraction proved completely ineffective.

Histamine and histamine liberators are not sufficient for eliciting the effect obtained with sensitizing antibody plus homologous antigen. It is postulated, therefore, that other effects occurring at the site of specific sensitization may also be responsible for the phenomenon.

INTRODUCTION

As reported previously, haemorrhagic reactions are produced in guinea-pigs at sites of passive cutaneous anaphylaxis (PCA) after intravenous injection of homologous antigen plus unrelated immune complex (Siqueira and Bier, 1961).

In view of the substantial work of Ishizaka and associates (reviewed by Ishizaka, 1963) on the biological activities of soluble antigen–antibody complexes and non-specifically aggregated γ-globulin, it was deemed of interest to investigate whether aggregated γ-globulin could replace the immune complex in the experimental model described by Siqueira and Bier (1961).

The results of these studies are presented in this paper together with complementary experiments aiming at the elucidation of the mechanism of the localizing effect of passive cutaneous sensitization.

MATERIALS AND METHODS

Antigens

Protein antigens were obtained from the following sources: bovine serum albumin (BSA), crystallized, obtained from Pentex, Kankakee, Illinois, U.S.A. and egg albumin (Ea), prepared in the laboratory by the method of Kekwick and Cannan as described by Kabat and Mayer (1961).

Antisera

Anti-BSA and anti-Ea were obtained from hyperimmunized rabbits prepared by a series of intravenous injections of alum precipitated antigen during a period of 60 days, totalling 60 mg. Weekly intramuscular injections of antigen incorporated in a mixture of paraffin oil and Arlacel A (incomplete Freund's adjuvant) were also used for immunization.

The antibody content of the antisera was determined by the quantitative precipitin method of Heidelberger and Kendall, as described by Kabat and Mayer (1961).

Protein determinations

Protein was estimated by the micro-Kjeldahl method, as described by Kabat and Mayer (1961). Alternatively, the biuret method and ultraviolet spectrophotometry were used.

Complement fixation

The number of units of complement (C′) fixed by each preparation of aggregated γ-globulin was determined by the method of Mayer, Oster, Bier and Heidelberger (1947).

Serial dilutions of the test samples were added to 100 $C'H_{50}$ (50 per cent units of C′) in a final volume of 10 ml and residual C′ activity was determined after incubation at 4° for 20 hours, in a final volume of 5 ml. The quantity of each preparation required for inactivation of 50 out of 100 $C'H_{50}$, $C'F_{50}$, was calculated according to Wallace, Osler and Mayer (1950).

Passive haemagglutination and passive haemolysis

A 2 per cent suspension of washed sheep erythrocytes was prepared in saline–phosphate buffer, pH 7·3, in which 3 mg/ml Ea had been previously dissolved. Ten millilitres of the red cell–antigen mixture were then incubated with 0·75 ml of bis-diazotized benzidine (0·37 mg/ml) for 20 minutes at room temperature and centrifuged at 1500 rev/min for 5 minutes. The erythrocyte sediment was washed three times and re-suspended in 20 ml of isotonic veronal buffer, pH 7·4, containing 0·00015 M $CaCl_2$, 0·0005 M $MgCl_2$ and 0·1 per cent gelatin.

For the passive haemagglutination tests 0·5 ml of serial dilutions of anti-Ea were incubated for 1 hour at 37° with 0·1 ml Ea-coated cell suspension. Readings were taken after one additional hour at room temperature. Controls included normal serum with coated and uncoated erythrocytes, as well as test serum with uncoated cells.

For the passive haemolysis tests, 0·5 ml of serial dilutions of serum were incubated for 30 minutes at 37° with 0·1 ml of coated cells plus 0·1 ml of a dilution of guinea-pig complement containing ten 50 per cent units per millilitre.

Fluorescein labelling

Purified HGG was dissolved in carbonate buffered saline, pH 9·5, chilled and mixed slowly, in the cold, with 0·06 mg fluorescein isothiocyanate per milligram of protein. After constant stirring overnight at 4°, the mixture was dialysed against phosphate buffered saline, pH 8, until no more fluorescein was visible.

The molar fluorescein: protein ratio was determined spectrophotometrically, fluorescein concentration being corrected for irrelevant absorption by subtracting one-half of the optical density (O.D.) value at 320 mμ from the O.D. value at 492 mμ (Beutner, Sepulveda and Barnett, 1968).

Aggregated γ-globulins

Human, horse, rabbit and bovine γ-globulins (HGG, HoGG, RGG and BGG) were prepared from normal sera by precipitation with ammonium sulphate at 0·5 saturation at pH 7·0 and purified by elution with 0·005 M phosphate buffer, pH 8·0, from a DEAE (diethylaminoethyl)-cellulose column. Eluates from segments corresponding to γ-globulin were pooled and mixed with an equal volume of saturated ammonium sulphate. The precipitate was dialysed against 0·16 M borate buffered saline, pH 8·0, and the solution was adjusted to a concentration of 20 mg/ml.

Aggregation was obtained by heating at 63° for 20 minutes, by mercaptoethanol–urea or by coupling with bis-diazotized benzidine, as described by Ishizaka and Ishizaka (1960).

Preparation of pepsin digested antibody

Purified γ-globulin, 2·4 g, obtained from rabbit anti-BSA was digested with 3 per cent (by weight) of pepsin at pH 4·5 for 18 hours at 37°. The digest was neutralized, dialysed for 48 hours against borate buffered saline, pH 8·0, and fractionally precipitated with sodium sulphate. The precipitate formed between 12·5 and 19 per cent sodium sulphate was re-dissolved in saline–borate buffer and dialysed for 72 hours against the same buffer.

Experimental model for the elicitation of haemorrhagic reactions at PCA sites

Albino guinea-pigs weighing 250 g ± 10 per cent are used. Animals are injected intradermally with 0·1 ml of an antiserum dilution corresponding to 2 μg Ab N/ml.

After a latent period of 2 hours, animals are challenged by the intravenous route with a mixture of the homologous antigen (0·2 mg N Ea or BSA) plus aggregated γ-globulin in a final volume of 1 ml.

Reactions are observed after 2 hours on the inner surface of the skin and the extent of haemorrhage recorded.

Each experiment should be accompanied by appropriate controls omitting the admixture of aggregated γ-globulin: in these animals haemorrhagic reactions are usually absent or minimal, although conspicuous haemorrhages are occasionally observed.

RESULTS

I. HAEMORRHAGE PRODUCING ACTIVITY OF HEAT-AGGREGATED γ-GLOBULINS OF DIFFERENT SPECIES

In agreement with previous findings on the biological properties of soluble antigen–antibody complexes, Ishizaka and Ishizaka (1959) have shown that human γ-globulin

aggregated by heating at 63° fixed complement and induced increased permeability of guinea-pig skin vessels, whereas the γ-globulins of horse, beef and chicken lacked both properties. Skin reactivity could never be elicited with aggregated γ-globulins derived from animal species whose antibodies are unable to sensitize guinea-pigs.

Apparently, the ability to become fixed to tissue and the formation of a toxic configuration associated with C′ fixation are essential prerequisites for the development of skin reactions under the experimental conditions of Ishizaka (1963).

In view of the results referred to above, experiments were performed with bovine, human, rabbit and horse γ-globulins aggregated by heat in order to compare their abilities to fix C′ and to elicit haemorrhagic reactions at PCA sites. As shown in Table 1, BGG had neither activities, whereas HGG, RGG and HoGG had both.

TABLE 1

HAEMORRHAGIC REACTIONS INDUCED AT PCA SITES BY HEAT-AGGREGATED γ-GLOBULINS OF DIFFERENT SPECIES

γ-Globulin species	Amount of i.v. injected γ-globulin (mg)					$C'F_{50}$ (mg)
	12	6	3	0·6	0·12	
Bovine			−	1+	tr	>2·4
	tr		−	tr	1+	
	1+		tr	tr	tr	
Human		3+	1+	3+	tr	0·03
		2+	3+	2+	1+	
		3+	2+	2+	tr	
Rabbit		4+				0·5
		3+				
		2+				
		1+				
Horse		2+	3+	1+	tr	0·3
		2+	4+	tr	2+	
		3+	3+	2+	tr	

II. HAEMORRHAGE PRODUCING ACTIVITY IN RELATION TO THE METHOD USED FOR AGGREGATION

While the complement fixing properties of aggregated γ-globulins do not depend on the method used for aggregation, aggregated HGG and RGG prepared by the mercaptoethanol–urea procedure as well as heat-aggregated RGG lack skin reactivity in spite of high C′ fixing ability (Ishizaka and Ishizaka, 1960).

It was, therefore, deemed of interest to investigate whether γ-globulins aggregated by different procedures would show differences in regard to haemorrhage producing activity. The results of these experiments are given in Table 2.

Human γ-globulin aggregated by heat or by mercaptoethanol–urea were equally effective in producing haemorrhagic reactions at PCA sites. Since the treatment of HGG with mercaptoethanol–urea results in preparations of low affinity for tissue, as shown in reversed PCA experiments (Ishizaka and Ishizaka, 1960), the structure involved in the fixation to skin does not seem to be required for the elicitation of haemorrhagic reactions at PCA sites. The same consideration applies to heat-aggregated RGG (cf.

Table 1) which is unable to induce skin reactivity under the experimental conditions of Ishizaka (1963).

In the experiments reported by Ishizaka and Ishizaka (1960) HoGG aggregated by coupling with bis-diazotized benzidine showed a weak C′ fixing ability and failed to induce increased capillary permeability in the guinea-pig skin. Our preparation of benzidine–azo-HoGG was strongly anti-complementary and produced conspicuous haemorrhagic reactions at PCA sites (Table 2). Here again, the ability to become fixed

TABLE 2

HAEMORRHAGIC REACTIONS INDUCED BY γ-GLOBULIN AGGREGATED BY DIFFERENT TECHNIQUES

γ-Globulin species	Technique used for aggregation	Intensity of haemorrhagic reactions			$C'F_{50}$ (mg)
Human	Heat	4+	4+	tr	0·03
Human	ME–urea	2+	4+	3+	0·10
Horse	BDB	3+	2+	3+	0·15

to tissue was not a prerequisite for the haemorrhage producing effect which, as in the other cases, was associated with the ability to fix C′.

The last relationship could also be shown by experiments with HGG with added α- and β-globulins, the mixture being aggregated by heat (Table 3).

TABLE 3

INFLUENCE OF CONTAMINATION WITH α- AND β-GLOBULINS ON THE ACTIVITY OF HEAT-AGGREGATED HUMAN γ-GLOBULIN IN REGARD TO C′ FIXATION AND TO THE INDUCTION OF HAEMORRHAGIC REACTION AT PCA SITES

Aggregated globulin	Haemorrhagic reactions after i.v. injection of 4 mg IgG				$C'F_{50}$ (mg)
Purified human γ	3+	2+	2+	2+	0·02–0·1
Mixture of human α, β and γG	tr	tr	1+	1+	>0·5

Purified HGG aggregated by heat became strongly anti-complementary ($C'F_{50}$ between 0·02 and 0·1 μg N) and showed marked haemorrhage producing activity. When contaminated by added α- and β-globulins and subjected to identical treatment, its $C'F_{50}$ value was much higher (more than 0·5 μg N) in accordance with the findings of Frommhagen and Fudenberg (1962) and the haemorrhage producing effect was practically nil.

III. INEFFECTIVENESS OF IMMUNE COMPLEXES PREPARED WITH PEPSIN DIGESTED ANTIBODY

The experiments reported in the preceding sections showed that the ability of aggregated γ-globulin to produce Arthus-like reactions at PCA sites was always associated with its capacity to fix C′. Since complement fixation is mainly a property of Porter fragment III (Taranta and Franklin, 1961; Ovary and Taranta, 1963), it was thought that immune

complexes prepared with pepsin-digested antibody, $F(ab')_2$, which lack the capacity to fix C′, should be inactive in regard to the elicitation of haemorrhagic reactions at PCA sites.

Antigen–antibody complexes prepared with untreated or pepsin-digested rabbit anti-BSA were compared at the same antibody level. As shown in Table 4, conspicuous

TABLE 4

COMPARATIVE EFFECTIVENESS OF IMMUNE COMPLEXES PREPARED WITH INTACT OR PEPSIN-DIGESTED ANTIBODY IN ELICITING HAEMORRHAGIC REACTIONS AT PCA SITES

Rabbit anti-BSA	F(ab′)2 fragment
4+	–
3+	–
3+	tr
1+	tr

haemorrhagic reactions were observed in the series corresponding to untreated antibody, whilst the immune complex prepared with $F(ab')_2$ proved completely ineffective.

IV. LOCALIZING ACTIVITY OF GUINEA-PIG γ_1 AND γ_2 ANTIBODIES

Guinea-pig precipitating antibodies belong to two classes of 7S immunoglobulins: the electrophoretically faster moving γ_1 and the slower moving γ_2 (Benacerraf, Ovary, Bloch and Franklin, 1963). The faster migrating antibody does not fix C′ and mediates

TABLE 5

HAEMORRHAGIC REACTIONS INDUCED BY AGGREGATED HUMAN γ-GLOBULIN AFTER SENSITIZATION WITH γ_1 AND γ_2 GUINEA-PIG ANTI-EGG ALBUMIN

Antibody* fraction	Haemagglutinating units of antibody			
	5120	1280	640	320
γ_1	4+	3+	4+	4+
	4+	4+	4+	3+
	4+		3+	4+
γ_2		tr		
		tr		
		1+		

* Passive haemagglutination titres of γ_1 and γ_2 fractions: 1:51,200 and 1:12,800 per ml, respectively.

anaphylaxis in the homologous species. The γ_2 antibodies do not sensitize the guinea-pig, but fix C′ and are far more efficient than γ_1 in provoking haemorrhagic necrosis in reverse passive Arthus experiments (Ovary, Benacerraf and Bloch, 1963).

The properties of guinea-pig γ_1 and γ_2 with regard to localization of haemorrhagic reactions were investigated. As for PCA, the ability to localize haemorrhagic reactions is an exclusive property of γ_1 (Table 5 and Fig. 1).

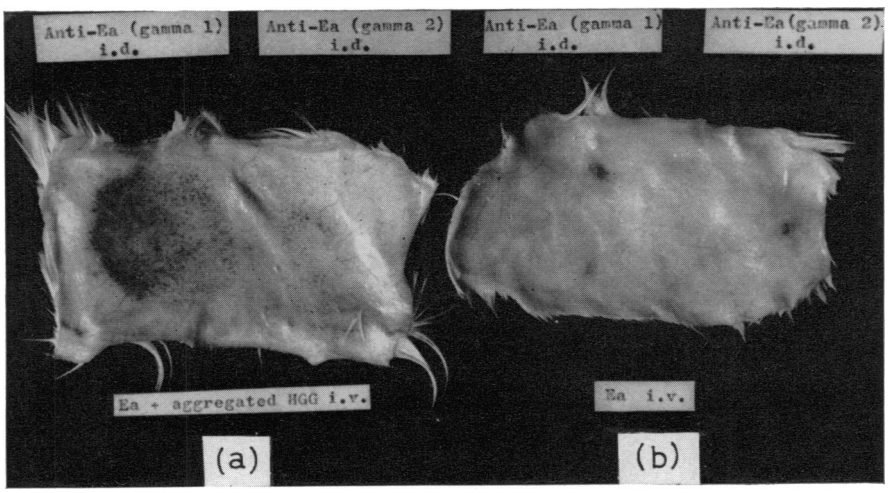

FIG. 1. (a) Left: Haemorrhagic reaction elicited at PCA site by intradermal injection of guinea-pig γ_1 anti-egg albumin followed 2 hours later by intravenous injection of egg albumin plus heat-aggregated human γ-globulin. Right: Negative reaction at site of intradermal injection of guinea-pig γ_2 antibody in the same animal. (b) Negative reactions in animal sensitized as above but challenged intravenously with egg albumin alone.

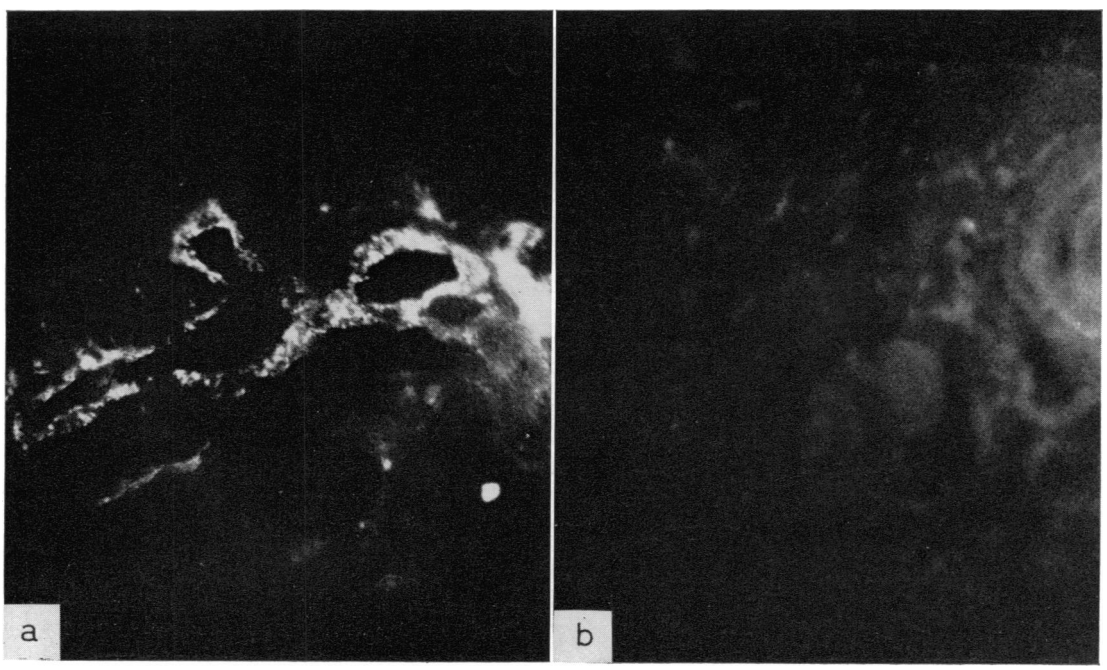

FIG. 2. (a) Localization of fluorescein labelled heat-aggregated γ-globulin at PCA site. (b) Negative result at a distant control site in the same animal. × 290.

(*Facing p.* 296)

DISCUSSION

The findings of the present investigation show that it is possible to transform the evanescent reaction associated to passive cutaneous anaphylaxis into a severe haemorrhagic reaction of the Arthus type by the intravenous challenge with homologous antigen plus non-specifically aggregated γ-globulin.

In the course of experiments on the production of nephrotoxic serum nephritis in decomplemented rats, Unanue and Dixon (1964) showed that animals receiving small, clinically ineffective doses of rabbit antikidney serum developed intense proteinuria when simultaneously injected with heat-aggregated HGG. This experimental model resembles the one used in our experiments and in both cases aggregated γ-globulin is demonstrable by fluorescence microscopy in the wall of vessels at sensitized sites (Fig. 2).

The affinity for tissue which is essential for skin reactivity under the experimental conditions of Ishizaka and Ishizaka (1960) does not play any role in the haemorrhage producing effect of aggregated γ-globulin under the conditions of the experiments herein reported. In fact, preparations such as heat-aggregated HoGG and mercaptoethanol–urea treated HGG which are unable to become fixed to the tissues of the skin are fully active in provoking haemorrhagic reactions at PCA sites. A common characteristic of all active preparations was the ability to fix C′, independently of the origin of the γ-globulin (human, horse and rabbit), or of the method used for aggregation. It would seem, therefore, that aggregated γ-globulin trapped in the wall of vessels at PCA sites act by a mechanism similar to that operating in the Arthus reaction, namely through C′ fixation followed by the formation of chemotactic factor, infiltration of polymorphonuclear leucocytes, phagocytosis of microprecipitates and presumable release of tissue-damaging lysosomal contents.

As to the localizing effect of PCA, its mechanism is still obscure.

Attempts to duplicate the effect of sensitizing antibody by intradermal injection of histamine or histamine liberators (dextran, 48/80 compound, anaphylatoxin, *Bothrops* venom), serotonin, bradikinin or mixtures therefrom, were uniformly negative. Apparently, histamine alone is not sufficient and requires adjuvant factors of cellular or humoral origin not considered in our experiments.

According to Movat, Lovett and Taichman (1966), there are two possible mechanisms operating in PCA in the rat, one involving the interaction of 'anaphylactic', mast-cell sensitizing antibody and another resembling a micro-Arthus reaction in its dependence on the phagocytosis of microprecipitates by polymorphonuclear leucocytes.

In the guinea-pig, Van der Berg, Oort and Rijssell (1962) have shown that in PCA reactions elicited by rabbit antibody, PMN-leucocytes appeared very early (after 5 minutes) after challenge with antigen.

In the experiments reported in this paper a micro-Arthus effect could hardly be made responsible for the localization of γ-globulin aggregates in the wall of vessels and subsequent development of vasculitis since the ability to localize haemorrhagic reactions was found to be an exclusive property of γ_1 and could never be obtained with the Arthus-producing γ_2. Furthermore, localization of haemorrhagic reactions at PCA sites which are easily observed in the guinea-pig could never be produced in the rat sensitized with rabbit antibody which provokes in this species a micro-Arthus type of reaction.

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