

From the Department of Pathophysiology and the Department of Medicine in Chest Clinic, University Medical School, Debrecen, Hungary.

THE LACK OF DETECTABLE IMMUNE COMPLEXES IN RAT ANAPHYLAXIS

By

L. FÉSÜS & M. KÁVAI

Providing a new insight into the pathomechanism of IgE-mediated systemic anaphylactic shock, severe alterations in the haemostatic mechanisms have been revealed in the rat during anaphylactic shock (4, 5, 7, 8). However, these alterations, i.e., the activation and consumption of Hageman factor, as well as the aggregation and partial disappearance of platelets from the circulation, could have been caused, at least in part, by circulating specific IgG antibodies through the intravascular formation of immune complexes (16). Therefore, it seemed to be reasonable to search for such material in rat anaphylaxis, for a further clarification of how the above mentioned processes could be mediated.

MATERIAL AND METHODS

Wistar male rats (150-200 g) were sensitized by an intraperitoneal injection of 3×10^{10} organisms of *Bordetella pertussis* vaccine (BPV) and by either 1 ml horse serum (HS) i.p. or 60 mg ovalbumin ($3 \times$ crystallized) i.m. Control animals (CA) received only BPV. Twelve days later blood was obtained with a plastic syringe from the abdominal aorta from one group of sensitized and untreated rats in deep pentobarbital anaesthesia (40 mg/kg pentobarbital i.p.), and from this, heparinized (10 IU heparin/1 ml blood) platelet rich plasma (HPRP) was prepared. For the preparation of test serum, blood was allowed to clot in a plastic tube and after 24 h at 4° C it was centrifuged at 2,000 g for

20 min. The other groups of sensitized animals were submitted to anaphylactic shock (AS) by the intravenous addition of 1 ml HS or 60 mg OA. CA were also given antigen. At various intervals, following antigen injection, blood was obtained as described above, allowed to clot in glass tubes, and sera were prepared.

Platelet aggregation was studied by Born's technique as described earlier (6).

Coagulation studies: 50 μ l antigen solution at various concentrations in pH 7.4 phosphate buffered saline (PBS) or preformed soluble immune complexes (IC) in PBS were added to 400 μ l of test serum (source of intact Hageman factor) in plastic tubes. After 20 min incubation at 37° C, 25- μ l aliquots were added to the prewarmed mixtures of 50 μ l phospholipid solution (1:50 dilution of 3.8 per cent Inositol in PBS) and 200 μ l lysozyme-treated substrate plasma (freshly prepared normal, citrated rat plasma to a 30 mg/ml lysozyme solution—an inhibitor of Hageman factor activation, see ref. 12.—in a ratio of 2:1) followed immediately by 50 μ l of 0.05 M CaCl_2 . The clotting times were recorded using a Clotek (Hyland) apparatus.

The radioassay of circulating immune complexes, a method based on the competitive inhibitory effect of soluble immune complexes on the ingestion of aggregated human immunoglobulins by guinea-pig peritoneal exudate cells, was carried out with a slight modification of the original procedure of Onyewotu et al. (13). Triplicate tests were set up in tubes containing 1×10^6 washed peritoneal macrophages in 0.5 ml Parker medium 199 to which 10 μ l of a 1:5 dilution of decomplexed sera in PBS (previously incubated at 56° C for 30 min and centrifuged at 1,000 g for 20 min) and 10 μ l of the aggregate solution (human IgG heated at 65° C for 15 min and centrifuged at 145,000 g for 1 h) labelled with ^{125}I (0.032 $\mu\text{Ci}/\mu\text{g}$ protein) by the lactoperoxidase technique (2) were added consecutively. After 75 min incubation at 37° C, the cells were washed three times by centrifugation and the radioactivity of the final suspended pellet was determined in a Packard β scintillation spectrometer. The results are expressed as the per cent inhibition of the binding of radioactivity (13).

Statistics. Mean values $\pm$ s.e. are indicated. Significance was assessed by Student's *t*-test.

RESULTS

A two-stage assay was used for measuring the activation of the contact phase of the coagulation system. Following the addition of various amounts of antigens to sera obtained from sensitized animals, no activation of Hageman factor could be observed (Table 1). When preformed soluble ICs (in antigen or in antibody excess) were applied, the activation of factor XII was obvious.

TABLE I
Results of Coagulation Studies.

Source of serum	Substances added to test serum	Clotting times	P
Control rats	PBS	129.9 $\pm$ 4.5	
	Celite, an activator of Hageman factor	36.4 $\pm$ 3.2	< 0.001
	IC (OA-rabbit anti-OA IgG, 10 μ g antibody 2 $\times$ antigen excess)	54.5 $\pm$ 6.7	< 0.001
	IC (OA-rabbit anti-OA IgG, 2.5 μ g antibody, 2 $\times$ antigen excess)	58.9 $\pm$ 6.9	< 0.001
Control rats BPV + HS sensitized rats	HS (no dilution)	85.4 $\pm$ 7.2	
	HS (no dilution)	83.4 $\pm$ 2.3	—
	HS (2 $\times$ diluted)	87.5 $\pm$ 3.1	—
	HS (2 $\times$ diluted)	92.3 $\pm$ 4.2	—
Control rats BPV + OA sensitized rats	OA (1 mg)	125.4 $\pm$ 5.2	
	OA (50 μ g)	127.5 $\pm$ 6.3	—
	OA (100 μ g)	128.0 $\pm$ 5.8	—
	OA (1 mg)	120.8 $\pm$ 3.1	—
Six animals in each group.			

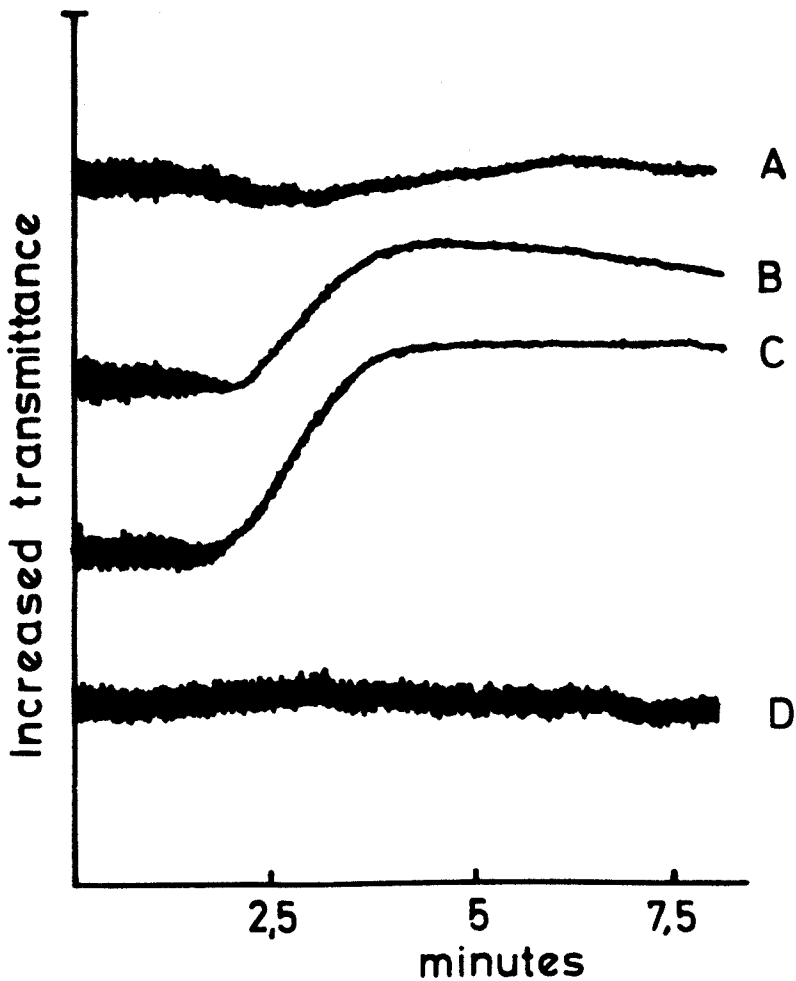


Fig. 1.

The changes of light transmittance through HPRP of normal rats following the addition of OA-rabbit anti-OA IgG ICs (A: 25 $\mu\text{g}/\text{ml}$ antibody, 2 $\times$ antigen excess; B: 25 $\mu\text{g}/\text{ml}$ antibody, 3 $\times$ antibody excess; C: 25 $\mu\text{g}/\text{ml}$ antibody, 2 $\times$ antibody excess) or various amounts of sera obtained 1, 2.5, 5, 10 min following anaphylactic challenge. (Curve D represents the common pattern of these experiments.) Platelet count: 500,000/ μl in each case (diluted by platelet poor plasma).

The addition of soluble ICs to HPRP resulted in platelet aggregation (Fig. 1). There was no aggregation when sera obtained at various intervals following the induction of AS were mixed with control HPRP (Fig. 1) or when various amounts of specific antigens were added to the HPRP of sensitized rats (not included in the Figure).

Using a highly sensitive radioassay capable of detecting

TABLE 2
Results of Immune Complex Determination.

Source of serum	IC (ng antibody)**	Per cent inhibition	P
Control human serum	—	6.1 $\pm$ 3.2	
Control rat serum	—	12.3 $\pm$ 8.2	
—	25.0	28.6 $\pm$ 5.6	< 0.001
—	50.0	46.7 $\pm$ 6.9	< 0.001
—	100.0	65.2 $\pm$ 10.0	< 0.001
AS* (antigen: HS) 1. min.	—	5.3 $\pm$ 2.5	—
2.5 min.	—	0.0 $\pm$ 0.15	—
5. min.	—	9.2 $\pm$ 5.3	—
10. min.	—	0.0 $\pm$ 2.6	—
AS* (antigen: OA) 1. min.	—	5.4 $\pm$ 3.8	—
2.5 min.	—	7.0 $\pm$ 6.2	—
5. min.	—	0.0 $\pm$ 3.4	—
10. min.	—	8.4 $\pm$ 6.3	—

* Four animals in each group.

** OA-rabbit anti-OA IgG, in a ratio of 20:1.

very low levels of ICs none of the anaphylactic sera was found to contain ICs (Table 2).

DISCUSSION

Anaphylaxis, the result of acute release of short-lived pharmacologically active agents when antigen is reintroduced, is conditioned mainly by the interaction of antigen with antibody fixed on tissues (9). In rats, the reaginic IgE antibody is formed in the early stage of primary immune response to protein antigen and BPV (with a maximum level between the 9th and 18th day) which then rapidly disappears (11). Therefore, systemic anaphylactic shock induced on the 9th–12th day is generally accepted as a cell-bound antibody-mediated reaction. However, the possibility of the appearance of specific “precipitating” IgG antibodies on the 9th–

12th day following such an immunization procedure, as a consequence of the formation of soluble ICs after the re-addition of antigen, cannot be entirely neglected. Jarret & Stewart found no such antibodies by the haemagglutination technique on the 12th day following the addition of BPV and antibody to Hooded Lister rats (10). On the other hand, Mota described low titres of precipitating antibodies in addition to reaginic ones on the 12th day in Wistar rats (11). Furthermore, it is currently thought that the anaphylactic γ Ga antibody in hyperimmune rat sera operates in the form of an antibody-antigen complex, which activates the complement system (14). The possible pathogenic role of IC has recently become clear since it has been shown that the activation of Hageman factor, the Hageman factor dependent pathways (4) and the aggregation of platelets (5) are factors involved in the development of severe anaphylactic symptoms. The initiation of these mechanisms could be mediated either by substances released from sensitized target cells or by the formation of circulating ICs following the readdition of antigen. To clarify this problem one has to examine whether detectable circulating ICs are formed in circumstances under which the above mentioned processes occur.

Three methods were used to search for ICs formed during AS or following the *in vitro* addition of specific antigen to the serum and to PRP of sensitized rats: a) In the *in vitro* coagulation studies the activation of Hageman factor by pre-formed ICs may be explained by the binding of Clq to the IC which exhibits a chemical composition similar to that of collagen (1). The addition of antigen to the serum of sensitized animals failed to produce activation, which means that no precipitating antibody was available for IC formation; b) It is generally accepted that ICs are able to aggregate platelets through various mechanisms (3). In our experiments there was no platelet aggregating material in the anaphylactic sera and it was not formed in the HPRP of sensitized animals following the addition of antigen; c) Using a method highly sensitive to the presence of ICs formed in a wide range of

antigen as well as antibody excess, IC could not be detected in the anaphylactic sera even at very low concentration.

Some possible explanations for these negative results may be: 1. The immunization procedure we used did not result in specific antibody production until the 12th day. 2. A low-rate production of such antibody began, but the formation of detectable IC did not occur because of the relatively high antigen dose used to induce AS. In any case, the role of immune complexes in the anaphylactic activation of Hageman factor and in the abrupt aggregation and disappearance of platelets during AS (4, 5) is excluded. Their role has also been questioned by the finding that the intrinsic coagulation is deeply altered in the anaphylactic shock of specifically immunized rabbits producing specific IgE exclusively (15). Consequently, another explanation of the initiation of anaphylactic platelet aggregation and Hageman factor activation must be found. Concerning platelet aggregation, the possible role of a "platelet aggregating factor" released during AS has been emphasized in rats too (5). For an exact explanation of the very early activation of Hageman factor and the Hageman factor-dependent processes further experiments are needed.

S U M M A R Y

Attempts were made to detect soluble immune complexes (IC) in sera of rats submitted to anaphylactic shock 12 days following BPV plus antigen treatment, and their *in vitro* formation following the addition of specific antigens to sera or heparinized platelet rich plasma of sensitized rats. We failed to detect ICs or IC formation by studying Hageman factor activation and platelet aggregation or by using a very sensitive radioassay of ICs. It is established that the anaphylactic activation of Hageman factor as well as the aggregation of platelets are not mediated by immune complexes, therefore, other explanations must be found.

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Authors' address:

Laszlo Fésüs, M.D.
National Institutes of Health
Bldg. 4, Room B1-10
Bethesda
Maryland, 20014
U.S.A.

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