

STUDIES ON THE LOCALIZATION OF CIRCULATING
ANTIGEN-ANTIBODY COMPLEXES AND OTHER
MACROMOLECULES IN VESSELS

II. PATHOGENETIC AND PHARMACODYNAMIC STUDIES*, †, §

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The accompanying paper (1) presented data concerning the position or resting place of antigen-antibody complexes in small vessel walls immediately after they leave the circulation under an anaphylactic provocation. The possible significance of this resting place in certain diseases of blood vessels was noted. The studies presented here were undertaken to investigate several different methods of injuring vessel walls that may provoke the localization of circulating complexes, and to study the pharmacologic background of each of these at least in part. These different methods of causing injury to the vessel included damage following the interaction of antigen and antibody in the vicinity of the vessels (systemic anaphylaxis), exposure to preformed anaphylatoxin, and direct attack by antibody directed against constituents of the vessel wall itself. It was hoped that studies such as these might help in the overall understanding of the mechanisms of localization of circulating macromolecules that, after their deposition, may bring about vascular damage.

Materials and Methods

Animals, antigens, and antibodies, with the exceptions noted below, were the same as those described in the adjoining article (1).

Antibodies against Forssman Antigen.—Anti-Forssman sera were obtained from rabbits following multiple injections of boiled sheep red blood cell stromata (2). Sera with antibody concentrations greater than 1:1024 were pooled.

Antibodies against Guinea Pig Platelets.—Guinea pig platelets were prepared from pooled plasma of heparinized guinea pigs (3). The antisera produced were repeatedly absorbed with pooled guinea pig leukocytes, red cells, and plasma.

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Pharmacologic Agents.—Histamine acid phosphate was obtained from Eli Lilly & Company, Indianapolis, 5-hydroxytryptamine (5-HT or serotonin) from Nutritional Biochemicals Corp., Cleveland; lysergic acid diethylamide (LSD) from Sandoz Pharmaceuticals, Hanover, New Jersey, chlorphenamine (chlortrimeton) from Schering Corp., Bloomfield, New Jersey; pyrilamine (neoantergan) and carbachol (carcholin) from Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania; octylamine (armeen 8D) from Armour Industrial Chemical Co., Chicago; and compound 48/80 from Burroughs Wellcome & Company, Inc., Tuckahoe, New York.

Anaphylatoxin.—The generic name anaphylatoxin is used here to represent the vasoactive material or materials released from fresh serum upon addition of antigen-antibody precipitates or other substances. Anaphylatoxin and heated control serum were prepared from fresh rat serum by the method given by Osler, *et al.* (4). Briefly, fresh rat serum was mixed with preformed, washed precipitates of Ea-anti-Ea (formed at the point of equivalence) in the ratio of 30 mg (Ab N) per ml of rat serum. The two were thoroughly mixed and incubated at 37°C for 90 minutes. After cooling to 0°C for 1 hour, the precipitate was centrifuged away at 12,000 g for 30 minutes at 0–4°C. The supernatant was used without dilution. Control serum was prepared simultaneously in exactly the same manner using the same rat serum, except the rat serum was heated to 56°C for 30 minutes prior to incubation with the immune precipitate.

Detection of Mast Cells in Tissues.—In order to demonstrate the presence of mast cells, it was found necessary to fix guinea pig tissues in 50 per cent ethanol containing 4 per cent lead subacetate according to the method of Mota (5).

Fluorescent Antibody Technique.—The fluorescent technique of Coons and Kaplan (6) was used and control tests as mentioned in the adjoining article were employed.

RESULTS

Several methods of bringing about the localization of circulating antigen-antibody were studied. As was noted in Table II of the accompanying paper, when guinea pigs with circulating complexes were subjected to anaphylactic shock using an unrelated antigen-antibody system, the circulating complexes became deposited in the walls of venules in many organs of the body. In addition to this method (classical passive systematic anaphylaxis) of provoking localization of the circulating complexes, several other methods of producing shock were tested.

1. *Localization Following Shock by Large Doses of Soluble Complexes.*—Injections of large doses of soluble complexes were also found to lead to the deposition of the same circulating antigen-antibody complexes. Twenty-five guinea pigs were injected intravenously with varying amounts of BSA-anti-BSA complexes, varying from 1.85 to 7.0 mg Ab N. Of these, 7 animals died of fatal anaphylaxis, 2 showed moderate anaphylactic symptoms, 3 mild, and the rest none. These results were similar to those reported by Germuth and McKinnon (7) and by us (8). Fluorescence revealed a marked vascular deposition of complexes in the fatal group, relatively less in the guinea pigs showing more mild anaphylactic symptoms, and none in animals without symptoms. Hence, it appeared that anaphylactic signs were a necessary concomitant to localization of circulating complexes and that when such signs were elicited, complexes would deposit in amounts roughly corresponding to the degree of anaphylaxis.

2. *Localization Subsequent to Shock Caused by Injections of Large Quantities of Sensitizing Antibody Followed by Challenge with Antigen.*—It was also possible to provoke localization of antigen and rabbit globulin by sensitizing guinea pigs with large quantities of anti-BSA and after 18 hours, challenging with BSA as noted in Table I. Those animals receiving 1.50 mg Ab N or greater as a sensitizing dose showed localization in venules following antigenic challenge.

Pharmacodynamic considerations: With the observation of the deposition in vessels of circulating macromolecules during the state of antigen-antibody anaphylaxis in the guinea pigs, it was deemed important to analyze, superficially at least, the pharmacologic cause or causes of the deposition. Hista-

TABLE I
*Localization of Antigen and Rabbit Globulin in Vessels of Sensitized Guinea Pigs Following Antigenic Challenge**

No. of guinea pigs	Sensitized anti-BSA Ab N	Challenging BSA (N)	Anaphylactic signs	Localization of BSA and RGG
	mg	mg		
3	2.50	1.0	Death	Moderate to marked
2	1.50	1.0	Death	Trace
3	0.50	1.0	Death	Neg.
3	0.10	1.0	Death	Neg.
3	0.03	1.0	Death	Neg.
2	—	1.0	Neg.	Neg.

* All animals challenged intravenously 18 hours following intravenous sensitization with antibody.

mine, 5-hydroxytryptamine (5-HT) and choline derivatives were evaluated. To accomplish this, use was made of specific inhibitors of the two vasoactive amines, histamine and 5-HT. Two antihistaminics, chlorphenamine (chlor-trimeton), and pyrilamine (neotanergan) were employed. As may be seen in Table II, it was found that anaphylaxis was inhibited by pretreatment with antihistaminics, as would be anticipated, and in addition, there was little or no observable deposition of the circulating complexes in vessel walls. The 5-HT inhibitor lysergic acid diethylamide (LSD) was employed in dosages up to 3 times that capable of completely blocking the lethal effects of 20 mg of 5-HT. This is many times the total quantity of 5-HT within the entire guinea pig. As shown in Table II, the large dosages of LSD were found incapable of blocking the anaphylactic localization of circulating complexes even when, using small dosages of antibody, mild anaphylactic stimulation was given. Attempts were also made to cause the localization of circulating complexes with intravenous injections of 5-HT, employing the same preparation and amount of complexes shown in Table II, and using up to 20 mg (superlethal dosage) of 5-HT. It was found that the injections of 5-HT failed to bring about more than a trace

localization in a small percentage of animals. In contrast, injections of 100 μg of histamine (base) brought about widespread localization in small vessels. This localization by extrinsic histamine resembled in all respects that following anaphylactic shock. In further experiments, the choline derivative carbachol was used to see if it was capable of causing deposition of circulating complexes. By injecting 200 μg carbachol intracutaneously, the guinea pigs underwent symptoms typical of systemic anaphylaxis, and had pulmonary changes in the gross and microscopically identical with that seen in anaphylaxis. However,

TABLE II
*Effects of Histamine and 5-Hydroxytryptamine (5-HT) Antagonists on the Anaphylactic Localization of Circulating Antigen-Antibody Complexes**

No. of guinea pigs	Antihistaminic		5-HT LSD (intramuscularly)	Soluble complexes (Ab N intravenously)	Anaphylactic signs	Vascular localization
	Chlorphenamine intravenously †	Pyrilamine (intramuscularly) §				
	mg	mg	mg	mg		
5	2.0	—	—	1.0	Neg.	Neg. or trace
7	—	1.0	—	1.0	Neg.	Neg. or trace
5	—	—	—	1.0	Death	Marked
7	—	—	0.25 ¶	1.0	Mild-mod.	Neg. to mod.
4	—	—	— ¶	1.0	Mild-mod.	Neg. to mod.
6	—	—	0.25	1.0	Death	Marked
10	—	—	—	1.0	Death	Marked

* All guinea pigs sensitized with 150 μg N anti-HGG and after 18 hours injected with soluble complexes and challenged with 1.0 mg N HGG, except where noted.

† Chlorphenamine injected 10 minutes prior to complexes and antigen.

§ Pyrilamine injected 30 minutes prior to complexes and antigen.

|| Lysergic acid diethylamide injected 30 minutes prior to complexes and antigen.

¶ Guinea pigs sensitized with 0.02 mg N anti-HGG and challenged with 1.0 mg N HGG.

when soluble complexes were injected intravenously simultaneously with the carbachol in 10 guinea pigs, only a trace or no localization of the complexes in pulmonary vessels occurred.

The results, then, strongly suggest that upon antigenic challenge of a sensitized guinea pig, in which soluble complexes were present in the circulation, that histamine was released in the tissues bringing about at least two events: (a) constriction of bronchial smooth muscle with subsequent anaphylactic death, and (b) alterations in the walls of small vessels leading to deposition of the circulating complexes.

Source of histamine responsible for the localization: With the abundant literature pointing to the mast cell as a source of vasoactive amines, correlative studies were undertaken to find if a relationship existed between the position of

vessels in which complexes would localize and the location of concentrations of mast cells in normal guinea pigs. The results showed that affected vessels at all times lay in positions where mast cells were grouped, namely in the connective tissue septa and about large vessels and structures that course through organs. Counts of mast cells in various organs revealed that within a radius of 0.17 mm of these structures there were up to 12 times as many mast cells as there were outside this zone.

Effect of mast cell disrupting agents on the localization of circulating complexes: Attempts were carried out to see if direct attack on the mast cells by agents known to cause release of histamine from these cells would bring about localization of the complexes. Two agents were used, octylamine and compound "48/80." Intravenous injections of 1 to 50 mg of octylamine in 19 guinea pigs brought about few symptoms of anaphylaxis in low dosages, but occasional symptoms and fatal collapse in higher dosage. Five out of 11 guinea pigs analyzed were noted to have had complexes localized in their pulmonary vessels. Only occasional vessels were involved in most instances, but they showed marked deposition as if maximally affected. Compound 48/80 was found to be ineffective in producing localization in dosages from 0.25 to 1.0 mg when given intravenously, even though guinea pigs collapsed and died at the larger dosage levels.

The role of platelets as a source of histamine in the deposition of circulating complexes: Attempts were made to find if circulating platelets, known to contain histamine and to release it upon contact with antigen-antibody complexes (9, 10), could be the reservoir of histamine responsible for the deposition of complexes. Accordingly, passively sensitized guinea pigs were depleted of platelets by injections of purified rabbit serum directed against guinea pig platelets and then challenged with soluble complexes and antigen. The results of both anaphylactic symptoms and localization of complexes are given in Table III. It may be seen that despite drastic or total elimination of platelets from the circulation, fatal anaphylaxis occurred uniformly upon antigenic challenge, and soluble complexes became localized equally as well as in untreated control guinea pigs. Sequestered platelets probably did not account for these findings, as fluorescent antibody techniques failed to demonstrate the presence of platelets in the pulmonary tissues of guinea pigs treated in the manner outlined.

3. *Anaphylatoxin Shock.*—Anaphylatoxin was prepared as noted above and injected into guinea pigs following the administration of soluble BSA-anti-BSA complexes. As noted in Table IV, rat anaphylatoxin was capable of provoking localization of circulating complexes and systemic shock typical of antigen-antibody anaphylaxis. In addition, the appearance by fluorescence microscopy of the complexes was identical with that in systemic anaphylaxis, and the distribution of affected vessels in lungs, heart, pancreas, gastrointesti-

nal tract, and renal pelvis was identical. Glomerular capillaries were uninvolved. Controls, in which preheated rat serum was used instead of fresh serum for exposure to immune precipitates showed mild anaphylactic symptoms in one out of four cases and an occasional pulmonary vessel with localized complexes in three out of four cases (all other organs tested failed to demonstrate positive vessels). Such activity might result from the formation of aggregated gamma globulin during heating either by direct action in the guinea pig or possibly by formation of some anaphylatoxin or other permeability factor during the heating process.

In an attempt to find if anaphylatoxin acted directly on the blood vessels or

TABLE III
*Effect of Platelet Depletion on Localization of Circulating Antigen-Antibody Complexes**

No. of guinea pigs	Platelet count‡	Complexes (N anti-BSA)	Anaphylactic signs	Vascular localization of complexes
	<i>mm³</i>	<i>mg</i>		
7	0 to 4100§	0.75	Death	Marked
3	203,000 to 383,000	0.75	Death	Marked

* All animals sensitized with either 150 μ g N anti-Ea or anti-HGG and simultaneously with rabbit antiplatelet antibody. Eighteen hours later guinea pigs were injected with a small quantity of antiplatelet antibody (see Materials and Methods) and after $\frac{1}{2}$ hour with complexes and 1.0 mg N Ea or HGG.

‡ Performed just prior to injection of complexes and challenging antigen.

§ Three of the 7 were entirely depleted of circulating platelets.

via mediators, the antihistaminic chlorphenamine was employed. As is noted in Table IV, chlorphenamine was found capable of completely or markedly modifying both systemic shock and localization of the circulating complexes following provocation by anaphylatoxin.

4. *Forssman Antibody Shock*.—Since the deposition of circulating complexes in the walls of vessels appeared to be occurring secondarily to some vascular injury or altered physiologic state, an attempt was made to see if direct insult to vascular tissue would result in localization. The agent chosen to produce the vascular injury was Forssman antibody, since Forssman antigen is common to vascular intima in guinea pigs (2). Accordingly, 8 guinea pigs were injected intravenously with soluble BSA-anti-BSA complexes followed within 15 seconds by 1.0 to 2.0 ml anti-Forssman antibody. After approximately 15 to 20 seconds gasping motions and occasional nose rubbing were noted. The guinea pigs staggered and fell in about 1 minute and death ensued. Strong signs of histamine shock such as lion-maned fur, sneezing and coughing, and strong inhalatory efforts were absent. Pulmonary distension was present at autopsy, however, much like that in anaphylaxis, but considerable amounts of paren-

chymal hemorrhages were present in addition. Microscopically, much edema and hemorrhage was observable in the alveolar spaces and about arteries although it was difficult to detect any convincing vascular damage. Using the fluorescent antibody technique, the complexes were found in the walls of occasional venules of the lung, heart, pancreas, and renal pelvis. Complexes could also be found extensively along the walls of alveolar capillaries as seen under high magnification.

In order to find whether the localization was caused by damage after antibody

TABLE IV
The Ability of Rat Anaphylatoxin and Forssman Antibody to Provoke the Localization of Circulating Soluble Antigen-Antibody Complexes in Guinea Pigs

No. of guinea pigs	Anaphylatoxin*	Rabbit Forssman antibody	Soluble complexes (N anti-BSA)	Antihistaminic (chlorphenamine)†	Systemic shock				Vascular localization of complexes
					Neg.	Mild	Sev.	Fatal	
7	2.0	—	0.50	—				7	Marked
6	2.0	—	0.50	1.0	4	2			Neg. to trace
4	2.0‡	—	0.50	—	3	1			Neg. to mild
7	—	1.0	0.50	—			2	5	Mod.
4	—	1.0	0.50	1.0				4	Mod.
2	—	—	0.50	—				2	Marked

* Prepared by exposing washed immune precipitates to fresh rat serum, see Materials and Methods.

† Chlorphenamine injected intravenously immediately before soluble complexes and challenging agent.

‡ Prepared as was anaphylatoxin, but from rat serum heated to 56°C, 15 minutes.

|| Control guinea pigs, passively sensitized with 150 µg N anti-Ea and after 18 hours injected intravenously with soluble complexes and challenged with 1.0 mg N Ea.

became bound to its antigen in the small vessels or whether again histamine played a role as mediator, 8 guinea pigs were given the antihistaminics chlorphenamine (2 mg intravenously) and pyrilamine (1 mg intravenously) prior to injections of complexes and rabbit anti-Forssman serum. Despite the antihistaminic, shock ensued upon challenge and antigen-antibody complexes localized as before. This was in marked contrast to the inhibitory effects of antihistaminics on the localization of complexes occurring during antigen-antibody or anaphylatoxin anaphylaxis.

DISCUSSION

These studies indicate that during systemic anaphylactic shock in guinea pigs caused by antigen and antibody there occurs an alteration in small vessel walls allowing circulating antigen-antibody complexes to localize. The state of

shock could be brought about by passive sensitization followed by challenge or by a single injection of a large dose of antigen-antibody complexes. The pharmacologic agent responsible for the alteration in the vessel walls was found to be histamine, the same agent responsible for the state of shock. Clearly 5-hydroxytryptamine and choline derivative did not bring about localization of circulating complexes in the guinea pigs. The source of the histamine in these studies was not unequivocally determined, although it may have been mast cells. By correlative studies mast cells were found to lie in close proximity to the affected vessels, and at least in some of the guinea pigs, the mast cell disrupting agent octylamine brought about localization. However, the correlation between site of mast cell concentration and affected vessels may be coincidental since it was found that exogenous histamine caused a similar pattern of involvement. This leaves open the possibility that histamine released in a single area of the body into the vascular stream could bring about permeability changes in vessels and deposition of complexes in distant organs. Platelets could be ruled out as a major source of histamine for both the deposition of complexes and anaphylaxis in the guinea pigs, thus confirming and extending the observations of Humphrey and Jaques (9). The finding that histamine may cause localization of circulating soluble complexes extends the earlier findings of Benacerraf, McCluskey, and Patras (10), who reported that injections of vasoactive amines or soluble complexes into mice brought about the deposition of circulating carbon particles in vessels and heart. They suggested that such reservoirs of vasoactive amines as mast cells could play an important role in the carbon localization.

The effect of the histamine on the vessel walls was probably one of increased permeability as determined by the ultrastructural studies reported in the accompanying article, and from the well known observations that histamine acts powerfully to increase vascular permeability. In relationship to known disease states, either human or experimental, it would seem possible, from morphologic observations, that the filtration of plasma by vessels normally or in a state of increased permeability could bring about localization of macromolecules. This would be true in such areas as the glomerulus and the endocardium in experimental serum sickness, where early deposits are clearly found between endothelium and basement membrane (11, 12), and perhaps in the necrotizing arteritis of serum sickness where similar deposits are frequently seen (13). Further work will be required, however, to determine whether such possibilities based on morphologic observations are justified by functional evidence.

Since, in experimental serum sickness in rabbits or in human diseases, severe generalized shock does not occur while complexes circulate, as it does in the guinea pigs, the localization of circulating complexes does not occur under

such extreme circumstances. However, it is certainly within the realm of possibility that release of vasoactive agents at a local level could occur from a reservoir either fixed at that site, *e.g.* a mast cell, or from a circulating reservoir, *e.g.* platelets, and bring about a local "shock" condition. With the resulting changes in the vessel walls, localization of circulating complexes could then result at that focus. In this regard, it is of interest that rabbit platelets have been shown to be agglutinated by complexes (14) which would allow sequestration to occur, and that rabbit platelets in the presence of antigen and antibody and fresh serum release vasoactive amines (15). Further studies have suggested a role of platelets in certain systemic immunologic diseases, although the mechanism of action is not yet clear (16).

The present studies on the biologic activity of anaphylatoxin would indicate that anaphylatoxin, when injected intravenously, will cause bronchial muscular constriction in guinea pigs and an alteration in venules generally, probably one of increased permeability. It is during this latter event that circulating complexes localized. These studies corroborate the data of Osler, *et al.*, (4) who demonstrated that anaphylatoxin produced from rat serum would cause increased vascular permeability in the area where it was locally injected and would bring about constriction of guinea pig ileum. In the present studies, the permeability and anaphylactic effects of rat anaphylatoxin were found to be mediated by histamine. This would suggest a link in the final pathway of the mediators in anaphylatoxin and anaphylactic shock in guinea pigs. A similar conclusion was drawn by Hahn and Oberdorf (17) and by Rocha e Silva and Aronson (18) by the findings that the effect of anaphylatoxin was blocked by antihistamines and that anaphylatoxin was capable of causing a release of histamine when perfused through guinea pig lung *in vitro*. Just how antigen and antibody or anaphylatoxin bring about the release of histamine is not known, although from the work of Mota (5, 19) both appear to disrupt mast cells. Whether the effect on mast cells of antigen and antibody may be mediated by anaphylatoxin is a subject of controversy.

The finding that antibody directed against Forssman antigen of the vessel walls also brought about localization of circulating complexes was not surprising in view of the above findings regarding the alteration of blood vessel walls. Vascular damage with consequent edema and hemorrhage following administration of the antibody were evident. Very little Forssman antigen was noted in the glomerulus and arteries, as opposed to the observations of Tanaka and Leduc (2), and perhaps as a consequence, little to no localization of complexes was found in these structures. The pharmacologic mediation of the damage by Forssman antibody was not elucidated in the present study although the action of histamine could be ruled out. Whether a parallel mechanism bringing about the localization of complexes exists in other experi-

mental models or in human disease conditions is not known. This would necessarily implicate the action of an autoantibody against vascular tissue rather than heterologous antibody as employed in the present study.

SUMMARY

Localization of circulating antigen-antibody complexes in vessels of guinea pigs by means of anaphylactic shock was found to be mediated by histamine that was released at the time of anaphylaxis. The source of the histamine may have been the mast cell as noted in studies employing a direct attack on the mast cells by octylamine. Platelets played apparently little to no role in guinea pigs in the anaphylactic deposition of circulating complexes.

Rat anaphylatoxin was found to cause vascular localization and symptoms of anaphylaxis identical with that brought about by antigen-antibody anaphylaxis. This also was found to be dependent upon the release of histamine.

Antibody against Forssman antigen in the vessel walls of the guinea pigs also led to deposition of circulating complexes. This was found not to be histamine dependent.

The possible role of local increase in vascular permeability in certain experimental disease states in the localization of circulating complexes is discussed.

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