ANTIGENICITY OF RAT COLLAGEN

Reverse Anaphylaxis Induced in Rats by Anti-Rat Collagen Serum*

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Plates 2 and 3

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In a previous report (1) from this laboratory it was shown that purified collagen prepared from the tail tendons of rats, little altered from the native state, is soluble in dilute acetic acid, and that repeated intraperitoneal injections of the collagen solutions induced complement-fixing antibodies in rabbits. Evidence was presented that these antibodies were directed specifically toward collagen. It was also demonstrated that soluble collagens prepared from rat tail tendon and from tunica of the carp swim bladder induced the formation of immunologically distinct antibodies, indicating that the native collagens probably have species specificity. Recently, additional evidence has been reported that soluble collagen prepared from another species, the chicken, also induced antibody formation in the rabbit, and in tissue cultures these antibodies have been found to be directed specifically toward chicken collagen (2).

Serum in which there are antibodies against blood or tissue antigens when introduced into the appropriate species of animal causes systemic anaphylactic shock or localized tissue damage (3). Thus, one may expect that the introduction into the rat of serum containing antibodies against rat collagen will cause either anaphylactic shock or injury to tissue collagen, or both. During the course of experiments designed to test these possibilities, it was discovered that serum from rabbits immunized with soluble rat collagen when injected intravenously into rats caused a reaction which appeared to be anaphylactic shock.

Since systemic anaphylaxis is one of the most sensitive indicators of the combination of antigen with antibody, it has been employed to identify unknown antigens and to demonstrate immunological specificity of proteins. For example, Lancefield (4) used

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anaphylaxis to study the antigenicity of various fractions from hemolytic streptococci; Seastone, Loring, and Chester (5) employed this method for detection of tobacco mosaic virus during purification procedures; and Rimington and Van den Ende (6) also used anaphylaxis to detect the presence of several protein antigens in normal horse serum. Such reports suggested that the use of anaphylaxis might make it possible to confirm and extend *in vivo* our earlier observations on the antigenicity of rat collagen.

The present report deals with experiments employing reverse anaphylaxis in the rat as a means to show that rat collagen is antigenic and immunologically different from fish collagen.

Materials and Methods

The preparation and purification of collagen from rat tail tendons and from the tunica of the carp swim bladder, the immunization of rabbits with soluble collagens for preparation of immune sera, and the technique of complement fixation tests have been described in an earlier communication (1). Young black and white hooded rats of the Whalen strain, of both sexes, weighing between 80 and 140 gm. were used. They were fed "big red" dog pellets,[1] bread, and milk daily and greens twice weekly with water *ad libitum*. All sera used had been heated to 56°C. for 30 minutes because preliminary experiments revealed intravenous injection of unheated anticollagen or normal rabbit sera induced transient weakness in rats. The rats were autopsied immediately after death. The tissues were fixed for microscopic examination in 10 per cent neutral formalin; paraffin sections were stained in all cases with hematoxylin and eosin, and on occasions also with Masson's trichrome stain for connective tissue, Weigert's differential stain for fibrin, Giemsa, Wright, or eosin–methylene blue. Other techniques employed will be described in the course of the experiments.

Experimental Observations

Effect of Intravenous Injection of Anti-Rat Collagen Serum in the Rat.—

To determine the effect of the anti-rat collagen rabbit serum, 24 animals were injected intravenously with 1.5 to 5.0 cc. of the antiserum, titrations of which for the estimation of antibody content had been made previously by complement fixation. The results of this experiment are shown in Table I. In all rats receiving 2.0 cc. or more of the antiserum a systemic reaction took place within 5 to 35 minutes after the intravenous injection. The rats became restless, moved about the cage, and the rate of respiration was increased. Weakness followed in a few minutes. The animals became quiet, flattened themselves on their bellies, and crawled about dragging their hindlegs. Tachypnea and dyspnea, with irregular respirations, were present at this stage; the rectal temperature dropped 3 to 7°F. below normal; and cyanosis was noted about the ears, toes, and tail. At times soft stools covered with bloody mucus were passed. The urine was not discolored. After 20 minutes or longer the weakness and prostration became more intense, and the animals lay on their sides. There was no response to stimulation. In most rats death occurred from 30 to 90 minutes after injection. In animals that survived, the weakness lasted as long as 120 minutes, after which the rats slowly became alert and next day appeared well. Rats surviving the shock were sacrificed 24 hours later for gross and histological examination. Of the 24 rats injected, 17 developed fatal, 5 non-fatal shock, and 2 showed no reaction. The 22 rats reacting with shock received 2.0 cc. or more of the antiserum, and the remaining 2 in which no reaction

[1] Distributed by Cooperative, G. L. F. Marketing Service Inc., Canandaigua, New York.

occurred received only 1.5 cc. The reaction was constant but varied in time of onset, duration, and intensity.

Having induced in rats a reaction similar to anaphylaxis with the anti-rat

TABLE I

Effect of Anti-Rat Collagen Rabbit Serum Injected Intravenously into Rats

Rat			Serum		Anaphylactic reaction			
						Onset	Duration	
No.	Sex	Weight	Volume	Antibody titer*	Degree of shock‡	Time after injection		Result
		gm.	*cc.*			*min.*	*min.*	
7-8	M	112	1.5	1:128	None	—	—	Survived
5-9	M	95	1.5	1:256	"	—	—	"
2-7	M	100	2.0	1:128	Severe	30	70	Died
2-8	M	102	2.0	1:128	"	25	60	"
1-7	F	95	3.0	1:128	Moderate	20	120	Survived
2-4	M	116	3.0	1:128	Severe	10	40	Died
7-6	M	110	3.0	1:128	Moderate	35	120	Survived
5-7	M	110	3.5	1:256	Moderate	30	115	"
9-1	F	105	4.0	1:128	Severe	10	60	Died
9-2	F	110	4.0	1:128	"	12	90	"
9-3	F	120	4.0	1:128	"	15	90	"
5-8	M	110	4.5	1:256	Moderate	30	110	Survived
3	M	123	5.0	1:128	Severe	7	33	Died
4	M	80	5.0	1:128	"	8	30	"
5	M	121	5.0	1:128	"	10	45	"
6	M	90	5.0	1:128	"	9	33	"
1-2	F	110	5.0	1:128	"	5	60	"
1-3	F	115	5.0	1:128	"	7	90	"
1-5	F	80	5.0	1:128	"	10	45	"
6-0	M	120	5.0	1:128	"	7	40	"
6-1	M	140	5.0	1:128	"	12	60	"
6-4	M	105	5.0	1:128	"	7	30	"
6-5	M	122	5.0	1:128	"	5	35	"
6-6	M	130	5.0	1:128	Moderate	10	115	Survived

* Antibody titer in all tables indicates highest dilution of antigen giving ++ or better complement fixation with undiluted serum.

‡ Severe shock indicates very severe prostration, dyspnea, weakness leading to death. Moderate shock indicates severe prostration, dyspnea, and weakness lasting 1 to 2 hours but no death.

collagen serum, the specificity of the reaction was demonstrated by the use of heterologous anti-fish collagen and normal rabbit serum as controls. The results of this experiment are summarized in Table II. It can be seen that, even though the antibody titer of the anti-fish collagen serum was much higher than that of the anti-rat collagen serum, as measured by complement fixation, the

animals developed no shock nor was there any in those receiving normal rabbit serum. These findings suggested that the reaction is referable to an antigen in the rat tissues which reacted with the antisera prepared in rabbits immunized with purified rat collagen and not with antisera to fish collagen.

Gross and Microscopic Findings.—Changes were apparent on opening the body cavities in rats with fatal shock or rats sacrificed within 24 hours after the non-fatal reaction.

The lungs filled the thoracic cavities but did not appear distended. Petechial hemorrhages were found over the pleural surfaces and occasionally over the pericardium and thymus. The liver was markedly enlarged, purple in color, firm, with rounded edges and a tense capsule. The spleen was enlarged and dark red. The most striking change was in the small intestine. Patchy and diffuse hemorrhage extended from the pylorus to the cecum, and the lymphoid tissue of Peyer's patches was covered with petechial hemorrhages (Fig. 2). Blood

TABLE II

Comparison of the Effect of Intravenous Injection into Rats of Anti-Rat Collagen, Anti-Fish Collagen or Normal Rabbit Serum

Serum injected			No. of rats	
Type	Antibody titer	Volume	Injected	Shocked
		cc.		
Anti-rat collagen rabbit serum	1:128	5	12	12*
Anti-fish collagen rabbit serum	1:1024	5	6	0
Normal rabbit serum	—	5	8	0

* All suffered severe shock; 10 died within 60 minutes, 2 survived shock lasting 120 minutes.

was often found in the lumen of the small intestine. Hemorrhages extended into the mesentery and were found in the mesenteric lymph nodes but the large intestine showed no gross change.

Histological examination showed more widespread vascular changes than seen in the gross. Erythrocyte thrombi were found in numerous small veins and capillaries, and rarely in small arterioles. The thrombi completely filled the dilated vessels; they consisted of a mass of packed red blood cells among which a few leukocytes were noted (Fig. 5). In rats suffering from shock of longest duration the erythrocyte outlines were lost, resulting in a homogeneous hyaline acidophilic appearance (Fig. 6). Fibrin stains revealed little if any fibrin and with the Giemsa or Wright stains only a few platelets were noted in these thrombi. Variation in staining qualities from pink to light blue with the hematoxylin and eosin stain was a striking feature.

In the heart, diffuse hemorrhages were frequently found between myocardial muscle fibers and at times there were erythrocyte thrombi in the myocardial veins and capillaries. The alveolar walls of the lungs were thickened, the capillaries congested and contained many erythrocyte thrombi (Fig. 7); the lumina of the alveoli contained extravasated red blood cells and a few leukocytes but little fibrin. Unusually large numbers of polymorphonuclear leukocytes were sequestrated in the capillaries of the widened alveolar walls (Fig. 8). Erythrocyte thrombi were found in many medium sized pulmonary veins and in a few small

arterioles. The bronchioles were dilated and epithelium flattened. The perivascular lymph spaces about the bronchioles and larger pulmonary vessels showed hemorrhages, edema, and increased numbers of eosinophiles. The liver showed intense congestion of the sinusoidal capillaries and portal veins, hemorrhages in the periportal connective tissue, and erythrocyte thrombi in the sinusoids but not as many as in the lung. Focal areas of necrotic hepatic cells with polymorphonuclear and lymphocytic infiltration were noted in some of the sections. The splenic pulp was markedly congested with blood but rarely were erythrocyte thrombi observed. The kidney showed focal hemorrhages between tubules. The glomerular capillaries were congested and a few showed erythrocyte thrombi but no fibrin. No changes were found in the basement membrane of the glomeruli. The lymph nodes and thymus also showed focal hemorrhages. In the intestine erythrocyte thrombi were found in the capillaries of the villi and in submucosal veins (Fig. 5 and 6). Many of the capillaries had broken walls with extravasation of blood into the lumen of the intestine and into the lymphoid tissue of Peyer's patches. Necrotic changes were found in epithelium of the villi (Fig. 4). Detailed studies with a variety of staining methods did not reveal any consistent abnormal change in the collagen of the connective tissue in various organs. Rats which survived the shock and were not sacrificed until 3 or more days had elapsed showed remarkable repair of the vascular damage with little change to be seen on gross or microscopic examination.

Control rats, injected with anti-fish collagen serum or normal rabbit serum, did show moderate congestion of blood in vessels of the liver, spleen, and lungs but no diffuse vascular injury with resultant petechiae or generalized hemorrhage throughout the tissues.

Changes in Cellular Elements of the Peripheral Blood Associated with Shock.— Since both the clinical reaction and the pathological findings appeared to be consistent with anaphylactic shock, it seemed of interest to study the effect of anti-rat collagen and anti-fish collagen rabbit sera on the cellular elements of the peripheral blood in rats.

In a series of 6 male rats weighing between 110 and 130 gm., erythrocyte, leukocyte, and differential counts and estimation of platelets were made prior to injection of the anticollagen serum. Into 3 of the rats 5.0 cc. of anti-rat collagen serum, titer 1:64, were injected intravenously and counts were made by standard hematological methods after 15, 30, 60, and 120 minutes. The shock produced was severe, causing death in the 3 rats shortly after the 120 minute interval. No significant changes were noted in the erythrocyte counts but the leukocytes dropped from an average of 16,500 to 7,100 per c. mm. during the first 15 minutes and remained in that range at 30 minutes, returned to the initial level at 60 minutes, and increased to about 20,000 range at 120 minutes. On differential counts the polymorphonuclears decreased from the 25 per cent range to about 5 per cent at the time of the leukopenia but with the leukocytosis they rose to 50 per cent of the total leukocyte count with a shift to young banded forms. The platelets were markedly reduced and clumped in all blood films made. At the 30 minute interval on some films none were seen. It is worthy of note that, during shock, bleeding from the tail was prolonged after specimens were obtained. Three rats injected with 5.0 cc. of anti-fish collagen rabbit serum showed no change in the erythrocytes, platelets, or blood coagulability but a definite leukocytosis to 36,000 per c. mm. with 60 per cent polymorphonuclears. No shock was noted in any of these rats.

These observations are added evidence of the specific effect of the anti-rat collagen serum which causes changes in the peripheral blood resembling those

described by other workers in anaphylactic shock, of other animal species; the leukopenia and thrombocytopenia have been regarded as an important feature of anaphylaxis (7, 8).

Changes in Serum Complement Levels with Shock.—Serum complement levels

TABLE III

*Rat Serum Complement Level before and after Intravenous Injection of Rabbit Anti-Rat Collagen or Anti-Fish Collagen Serum**

Rat No.	Time relation to antiserum injection	Volume of rat serum, diluted 1:7							
		0.2 cc.	0.16 cc.	0.13 cc.	0.10 cc.	0.08 cc.	0.065 cc.	0.05 cc.	No serum cell control
Rats injected with 4 cc. of rabbit anti-rat collagen serum, antibody titer 1:64									
1	Before	++++	++++	++++	++++	++++	++++	++	
	After‡	++++	++++	++++	++++	+++	++	+	−
2	Before	++++	++++	++++	++++	++++	+++	+±	
	After‡	++++	++++	+++	++	+	+	−	−
9-4	Before	++++	++++	++++	++++	++++	+++	+±	
	After‡	++++	++++	++++	+++	++	−	−	−
9-5	Before	++++	++++	++++	++++	++++	++++	++	
	After‡	++++	+++	++	+	−	−	−	−
Rats injected with 4 cc. of rabbit anti-fish collagen serum, antibody titer 1:1024									
9-7	Before	++++	++++	++++	++++	++++	+++±	++	
	After‡	++++	++++	++++	++++	++++	+++	++	−
9-8	Before	++++	++++	++++	++++	++++	+++	++	
	After‡	++++	++++	++++	++++	++++	+++	++	−

* Complement level of blood is expressed in terms of the amount of hemolysis; ++++ indicating complete hemolysis to − indicating no hemolysis occurring in these systems under standardized conditions.

‡ Samples were taken 3 hours after antiserum injection.

have also been found to be lowered in active and passive anaphylaxis (9). In the present study the serum complement levels were determined in 6 male rats before and 3 hours after intravenous injections of anticollagen serum.

Blood was obtained by intracardiac puncture and placed in silicone lined tubes. After clotting at room temperature for 20 minutes, the tubes were centrifuged for 10 minutes at 2500 R.P.M. and the clear supernatant serum poured off. The serum was frozen immediately and kept in a CO_2 ice box at −72°C. until used. After waiting 14 days to allow the rats to recover completely from the cardiac puncture, 4 were injected with 4.0 cc. of anti-rat collagen

serum, titer 1:64, and 2 with 4.0 cc. of anti-fish collagen serum, titer 1:1024. The rats injected with the anti-rat collagen serum developed moderate shock 30 to 38 minutes after injection and the shock lasted from 120 to 145 minutes; the rats receiving the anti-fish collagen serum showed no ill effects. About 3 hours after the injection, blood was obtained by cardiac puncture from both groups of rats. Serum was collected as previously mentioned and stored in the CO_2 ice chest at $-72°C$. Complement levels of serum were tested after diluting 1:7 in veronal saline buffer. Each serum before and after shock was tested with decreasing concentrations, as shown in Table III, made up to a constant volume of 1.0 cc. with the buffer containing traces of magnesium and calcium (10), and 0.5 cc. of a 3 per cent suspension of sensitized sheep erythrocytes in buffer was added to make a total volume of 1.5 cc. After incubation in a water bath at 37°C. for 30 minutes and at room temperature for 10 minutes, the tubes were read to determine the amount of hemolysis. The complement levels are expressed in terms of the degree of hemolysis.

From the findings shown in Table III, complement levels appear to be distinctly lower after the shock in rats injected with the anti-rat collagen serum but essentially unchanged in those receiving anti-fish collagen serum. This reduction in serum complement together with the leukopenia and thrombocytopenia in rats undergoing shock strongly supports the view that this reaction is anaphylactic shock, and points to the specificity of the anti-rat collagen serum as compared with the anti-fish collagen serum.

Fractionation of Anti-Rat Collagen Rabbit Serum.—The data thus far presented suggest that the anaphylactic factor is an antibody relatively specific for rat collagen. Additional evidence that the reacting factor is antibody was sought by attempting to demonstrate its presence in the gamma globulin fraction of the serum.

Anti-rat collagen rabbit serum, titer 1:128, and normal rabbit serum were inactivated at 56°C. for 30 minutes and fractionated according to the method described by Lippman, Marti, and Campbell (11). These workers found that rabbit sera precipitated by one-third saturation with ammonium sulfate yielded a precipitate which electrophoretic analysis showed to be composed entirely of gamma globulin. To 30 cc. of the inactivated anti-rat collagen or normal rabbit sera was added 15 cc. of saturated ammonium sulfate, pH 7.8, and the mixtures were allowed to stand for 30 minutes at room temperature with occasional shaking, then centrifuged, and the supernates removed. Each precipitate was dissolved in 3 cc. of physiological saline. Each supernate and precipitate was placed in a collodion sac and dialyzed at 4°C. for 60 hours against frequent changes of physiological saline until the dialysate was free of sulfate ions. The supernate fraction of each serum was adjusted with physiological saline to 30 cc., and the precipitates to 6 cc. (concentrated 5-fold). This provided a supernatant protein fraction and a precipitated gamma globulin fraction from both anti-rat collagen and normal rabbit sera for injection into rats to determine in which fraction the active component was present. A group of male rats weighing between 100 and 120 gm. was injected intravenously with each of the 4 fractions: 4 rats each received 5 cc. of the supernatant protein fraction, and another 4 each received 1 cc. of the gamma globulin fraction from normal rabbit serum; 4 rats were each injected with 5 cc. of the supernate, and 6 with 1 cc. of the gamma globulin from the anti-rat collagen serum. Only the 6 rats receiving the gamma globulin fraction of the anti-rat collagen serum developed shock; in 4 of these it was severe and fatal; in the remaining 2 it was moderate and did not cause death. The shock appeared

within 20 minutes in all and lasted 60 minutes in those which died and 90 minutes in the 2 which survived. The surviving rats were sacrificed 24 hours later for postmortem studies. Typical gross and histological diffuse vascular injury was found in all the animals undergoing shock but not in any of the others.

Since the supernatant protein fractions of both anticollagen and normal rabbit sera and the gamma globulin fraction of the normal rabbit serum had no anaphylactic effect as far as could be determined by clinical or morphological observations, it appears that the reacting substance is produced by immunizing

TABLE IV

Correlation of Antibody Titer with Degree of Shock and Vascular Injury in Rats Intravenously Injected with 5.0 cc. of Anti-Rat Collagen Rabbit Serum

Rat			Serum antibody titer	Anaphylactic reaction				Vascular injury‡
				Degree of shock*	Onset	Duration	Result	
No.	Sex	Weight			Time after injection			
		gm.			*min.*	*min.*		
7-9	F	110	1:2	None	—	—	Survived	−
8-0	"	125	"	"	—	—	"	±
8-1	"	127	"	Mild	32	15	"	+
8-2	"	115	1:8	Moderate	30	120	"	++
8-3	"	120	"	"	25	106	"	++
8-4	"	110	"	"	20	118	"	+++
8-5	"	110	1:16	Severe	15	55	Died	++
8-6	"	120	"	"	12	108	"	++++
8-7	"	125	"	"	10	110	"	+++

* Mild shock indicates mild prostration, weakness, or dyspnea, but no death. Moderate shock indicates severe prostration, weakness, and dyspnea lasting 1 to 2 hours, but no death. Severe shock indicates very severe prostration, weakness, and dyspnea leading to death.

‡ Rough estimate of extent and intensity of gross and microscopic vascular injury indicated from − to ++++.

rabbits with rat collagen and it is present in the gamma globulin fraction of the serum protein. It is also noteworthy that shock produced with 1 cc. of the gamma globulin concentrate was as intense and severe as with 5 cc. of whole serum.

Relation of Antibody Titer to Degree of Shock and Vascular Injury.—The degree of passive anaphylaxis has been shown by Doerr and Russ (12) to be dependent upon the antibody content, as determined by precipitin reactions; and the amount of antibody required for passive sensitization has been quantitated by Kabat and Boldt (13). These reports suggested that a study of reverse anaphylaxis might show a relationship between complement-fixing antibodies and the capacity of the serum to induce shock.

A group of 9 female rats of relatively uniform weight were injected intravenously with 5.0 cc. of anti-rat collagen rabbit serum of varying titers as determined by complement fixation tests. The results of this experiment, shown in Table IV reveal a relatively constant relationship between antibody titer, degree of shock, and extent of pathological vascular injury as shown by gross and microscopic study. The rats surviving shock were sacrificed shortly after they appeared to have definitely recovered. All 3 animals receiving serum of 1:16 titer died and showed vascular injury more intense than the 3 which received the serum having a titer of 1:8 and far greater than those animals injected with the serum of only 1:2 titer. The differences among the 3 groups of rats were more distinct from clinical observation than from histologic study.

Although no final conclusions can be drawn from an experiment employing so few animals, the findings suggest that the content of specific antibody determines the intensity of the shock and resultant pathological abnormalities.

TABLE V A

Complement Fixation Test Following Absorption of Anti-Rat Collagen Rabbit Serum with Sheep or Rat Erythrocytes

Antiserum prepared against purified rat collagen	Antigen: purified rat collagen, 1 mg./cc. diluted 1:										
	1	2	4	8	16	32	64	128	256	512	1024
Absorbed with sheep erythrocytes	++++	++++	++++	++++	++++	++++	++++	++++	++	±	−
Absorbed with rat erythrocytes	++++	+++±	++++	++++	++++	++++	++++	++++	+++	±	−
Unabsorbed: control	++++	++++	++++	++++	++++	+++	+++	+++	+	±	−

− indicates complete hemolysis; ± to +++ indicates intermediate degrees of hemolysis; and ++++ indicates no hemolysis with complete fixation of complement.

Effect of Repeated Injections of Anti-Rat Collagen Serum.—In an effort to induce specific collagen injury *in vivo*, a group of 10 male and female rats were injected intravenously with anti-rat collagen rabbit serum, titer 1:128. All survived the initial shocking reaction and received subsequent injections equal to or greater than the initial. Although no specific injury to the collagen was demonstrated, a definite refractory state characteristic of anaphylaxis was observed.

A variety of dosage schedules were employed. Five rats were injected with 2.0 cc. of the anti-rat collagen serum on 5 successive days and were sacrificed from 3 to 10 days after the last injection. Moderately severe shock occurred after the first dose but no reaction was noted on subsequent injections. Two rats received 2.0, 2.5, 2.5, and 2.5 cc. of antiserum on consecutive days, and were sacrificed 13 days following the last injection; again shock occurred only after the initial injection. One rat was injected with 3.0 cc. following which shock developed but 3 days later received 5.0 cc. without effect; it was sacrificed 7 days later. The remaining 2 rats were injected with 2.0 cc.; both were shocked. Then on successive days they received 2.5, 3.0, 3.5, 4.0, 4.5, and finally 5.0 cc. In both of these animals only the 5.0 cc. caused any effect, weakness and increased respiratory rates for 10 to 15 minutes were noted, and no diffuse vascular injury was noted in any of these animals except

some pulmonary capillary congestion in those which had received 5.0 cc. No injury to collagen, however, could be demonstrated.

These findings suggest that the vascular changes in shocked rats are rapidly repaired, and that rats which survive anaphylaxis pass into a temporary refractory state after which they may again become susceptible to the reaction.

TABLE V B

Effect of Intravenous Injection into Rats of 5.0 cc. of Anti-Rat Collagen Rabbit Serum Absorbed with Sheep or Rat Erythrocytes

Rat			Serum		Anaphylactic reaction			
No.	Sex	Weight	Absorbed with:	Antibody titer	Degree of shock*	Onset (Time after injection)	Duration (Time after injection)	Result
		gm.				*min.*	*min.*	
7-3	M	130	Sheep erythrocytes	1:256	Severe	20	70	Died
7-4	M	125	"	"	"	25	90	"
7-5	M	122	"	"	"	18	80	"
8-8	F	125	"	"	Moderate	11	105	Survived
8-9	F	120	"	"	Severe	15	60	Died
9-0	F	115	"	"	"	20	60	"
2-0	M	107	Rat erythrocytes	"	"	16	30	"
2-1	M	120	"	"	"	10	45	"
5-3	M	96	"	"	"	5	60	"
5-4	M	101	"	"	"	5	120	"
5-5	M	105	"	"	"	10	80	"
5-6	M	107	"	"	Moderate	20	118	Survived
10-2	M	110	Unabsorbed	"	Severe	30	55	Died
10-3	M	120	"	"	"	7	40	"
10-4	M	140	"	"	"	12	60	"

* Severe shock indicates very severe prostration, dyspnea, and weakness leading to death.

Moderate shock indicates severe prostration, dyspnea, and weakness lasting 1 to 2 hours but no death.

Absorption of Anti-Rat Collagen Rabbit Serum with Sheep or Rat Erythrocytes.— The reaction induced in rats by an injection of anti-rat collagen serum, although correlated with the titer of specific complement-fixing antibodies, might have been due to heterogenetic antibody or to antibody against rat erythrocytes present in the collagen preparations. It appeared unlikely that it could be due to Forssman antigen-antibody system because rat tissues do not contain this antigen. The following experiments were done to test these possibilities.

Aliquots of anti-rat collagen serum inactivated at 56°C. for 30 minutes were mixed with sheep or rat erythrocytes, previously washed 3 times in veronal saline buffer (pH 7.4), in the proportion of 3 parts serum to one part packed erythrocytes, incubated in a water bath

at 37°C. for 30 minutes, and then stored overnight at 4°C. After centrifugation the clear supernatant serum was removed. Samples of the sera in the unabsorbed state and after absorption with sheep or rat erythrocytes were tested by complement fixation and by injection into rats. Table V A demonstrates no deterioration of the complement-fixing antibodies by absorption with sheep or rat erythrocytes, and perhaps more significantly as shown in Table V B the production of reverse anaphylaxis in rats was not altered by absorption of the serum.

From these experiments it is apparent that neither a heterogenetic antigen common to sheep erythrocytes nor antibodies to rat erythrocytes played a role in the anaphylactic shock.

Absorption of Anti-Rat Collagen Rabbit Serum with Native Rat or Fish Collagen.—It seemed desirable to ascertain whether the ability of anti-rat collagen rabbit serum to produce shock after injection into rats would be altered by

TABLE VI A

Complement Fixation Tests Following Absorption of Anti-Rat Collagen Rabbit Serum with Native Rat or Fish Collagen

Antiserum prepared against purified rat collagen	Antigen: purified rat collagen 1 mg./cc. diluted 1:										
	1	2	4	8	16	32	64	128	256	512	1024
Absorbed with rat collagen	−	−	−	−	−	−	−	−	−	−	−
Absorbed with fish collagen	++++	++++	++++	++++	++++	++++	++++	++++	+	+	−
Unabsorbed: control	++++	++++	++++	++++	++++	++++	++++	++±	±	−	−

− indicates complete hemolysis; ± to +++ indicates intermediate degrees of hemolysis; and ++++ indicates no hemolysis with complete fixation of complement.

absorption with homologous or heterologous collagen. This experiment appeared to be particularly pertinent in determining whether species specificity of the collagens exists and whether the antibodies are directed to collagen.

Aliquots of pooled anti-rat collagen rabbit serum were mixed with thoroughly washed finely minced native rat or fish collagen in the proportion of 3 parts serum to 1 part packed collagen, (30 cc. of serum to 10 gm. collagen, wet weight). The mixtures were incubated for 2 hours at 37°C., stored overnight at 4°C., and centrifuged at 3,500 R.P.M. in the cold to remove large particles of collagen and then centrifuged again for 90 minutes at 40,000 R.P.M. at 0°C. Samples of these sera were tested in the absorbed and unabsorbed states both by complement fixation and by intravenous injection into rats.

From the results shown in Table VI A complete absorption of the complement-fixing antibodies by the native rat collagen took place but the heterologous fish collagen failed to alter the antibody titer. More striking are the results in Table VI B demonstrating no reaction in rats injected with 5.0 cc. of anti-rat collagen rabbit sera absorbed with the native rat collagen but severe to moderate anaphylactic shock in all animals receiving a similar amount of the serum

absorbed with heterologous fish collagen or the unabsorbed control serum. All animals were autopsied, those that recovered from shock being sacrificed 18 hours after the injection. Vascular injury was demonstrated by gross and histological examination of the shock animals, but only congestion of pulmonary capillaries was found in the non-shocked rats.

TABLE VI B

Effect of Intravenous Injection into Rats of 5.0 cc. of Anti-Rat Collagen Rabbit Serum Absorbed with Native Rat or Fish Collagen

Rat			Serum		Anaphylactic reaction			
No.	Sex	Weight	Absorbed with:	Antibody titer	Degree of shock*	Onset (Time after injection)	Duration (Time after injection)	Result
		gm.				*min.*	*min.*	
1-6	M	130	Rat collagen	0	None	—	—	Survived
6-7	M	125	" "	0	"	—	—	"
6-8	M	130	" "	0	"	—	—	"
6-9	M	128	" "	0	"	—	—	"
1-4	M	140	Fish collagen	1:128	Severe	12	32	Died
7-0	M	125	" "	1:128	"	7	50	"
7-1	M	128	" "	1:128	"	12	45	"
7-2	M	129	" "	1:128	Moderate	15	120	Survived
10-5	M	105	Unabsorbed	1:128	Severe	8	120	Died
10-6	M	122	"	1:128	"	5	90	"
10-7	M	130	"	1:128	Moderate	14	118	Survived

* Severe shock indicates very severe prostration, dyspnea, and weakness leading to death. Moderate shock indicates severe prostration, dyspnea, and weakness lasting 1 to 2 hours but no death.

DISCUSSION

The observations presented in this paper show that the rat reacts to the intravenous injection of anti-rat collagen rabbit serum with symptoms and lesions characteristic of anaphylaxis in this species (14). The clinical and pathological findings in rats originally reported by Parker and Parker (14 *a*) in direct anaphylaxis, and by Smadel and Swift (15) in reverse anaphylaxis are essentially the same as herein described. Additional evidence of the anaphylactic nature of the reaction was shown by the transient leukopenia with rebound leukocytosis, thrombocytopenia, and diminished blood coagulability. The decrease in serum complement titers in rats 3 hours after injection of the antibody causing the reaction was also suggestive of anaphylaxis. A similar drop in titer of complement has been shown to occur in guinea pigs during anaphylactic shock (9). The refractory state developing after initial shock with sublethal amounts of the antibody is likewise in accord with anaphylaxis and

may be related to the lowering of the complement level. However, systematic studies to correlate the refractory state with complement titer were not carried out.

Among the microscopic findings in rats examined after shock, the erythrocytic thrombi were particularly noteworthy. These thrombi, found also in conditions other than anaphylaxis and first described by Flexner (16), are apparently the result of a change of unknown nature that occurs *in vivo*. Presumably there is no direct interaction between antibody and erythrocyte in the host because in the test tube the antibody was not absorbed by rat erythrocytes. Also of interest was the sequestration of polymorphonuclear leukocytes in the small blood vessels of the lungs coincident with the transient leukopenia and persisting during the subsequent leukocytosis. A similar relationship between trapping of leukocytes in the lungs and leukopenia had been noted previously by Andrewes (17) after injection of bacterial antigens into immunized rabbits, and by Webb (18) employing horse serum in dog anaphylaxis.

In spite of the fact that the reaction in rats appeared to be anaphylactic in character, it was essential to determine whether or not it was produced by a specific reaction of anti-rat collagen antibody in the serum with native collagen. The injection of heated normal rabbit serum or heated rabbit serum containing antibodies to fish collagen of very high titer caused no shock; this indicated the specificity of the reaction with anti-rat collagen serum. More significantly, an absorption of antibody from potent serum by homologous rat collagen removed the shock-producing factor, whereas absorption with heterologous fish collagen left both the titer and potency undiminished. These findings lend weight to the thesis that a specific anti-collagen antibody reacts with collagen in the host and induces the anaphylactic reaction. In control experiments, a number of other possibilities were excluded: the Forssman antigen is not contained in rat tissue and consequently could not have induced Forssman antibodies in the immunized rabbits; antibodies to rat erythrocytes or to antigens in sheep erythrocytes other than Forssman were removed by absorption without reduction in the anaphylactic potency of the serum.

The failure to demonstrate injury to collagen in rats injected with lethal or sublethal amounts of anti-collagen serum can not as yet be explained. Rats which received lethal doses may have survived too short a time for specific collagen injury to develop, or the diffuse vascular reaction may have obscured damage to the collagen fibers. Since collagen is widely distributed throughout almost all tissues, antibody directed toward such an antigen may lead only to a systemic anaphylactic reaction rather than to inducing focal specific injury such as that described by Smadel (19) in the kidney after injecting anti-rat kidney antibody. Another factor which may play a role in the failure to demonstrate collagen injury is the relatively low antibody content of the sera employed, or a disproportion between antibody and antigen. Evidence, how-

ever, is available that antibody to collagen does cause abnormal changes to the collagen fiber. Sera containing antibodies which fix complement in the presence of homologous rat collagen also interfere with the reconstitution from solution of normal rat tail collagen fibers (1). Moreover, antiserum to purified chicken collagen has an inhibitory action on collagen fibrogenesis in tissue cultures of the chick dermis (2).

It is possible that the observations of Kay (20) on the production of experimental nephritis in rabbits by the use of anti rabbit kidney duck serum may be pertinent to the problem of inducing specific injury to collagen in the rat. He found that antibody in the duck serum formed an apparently harmless combination with antigen in the rabbit kidney but that 3 to 5 days later the rabbit developed antibodies to duck serum which then by reacting with the antigen-antibody complex previously formed in the kidney caused nephritis. If this sequence of events applies, the poor antibody-forming ability of the rat may also contribute to the absence of collagen injury. Perhaps the fluorescent antibody technique described by Coons (21, 22) may make it possible to establish the site of antibody-antigen reaction and to concentrate further search for collagen injury at these points.

Although no injury to collagen has been shown to occur in the rat, the present study demonstrated specific reverse anaphylactic shock and confirms the evidence previously reported on the antigenicity and species specificity of rat collagen.

SUMMARY AND CONCLUSIONS

Reverse anaphylactic shock was induced in rats by intravenous injection of serum containing complement-fixing antibodies, obtained by immunization of rabbits with purified preparations of rat tail collagen. Normal rabbit serum or serum containing antibodies to collagen from tunica of carp swim bladder was without effect. The clinical and pathological findings resembled those described by previous workers studying direct and reverse anaphylactic reactions induced in the rat with other antigens. Thrombocytopenia, leukopenia with rebound leukocytosis, delayed blood coagulability, lowering of serum complement and the development of a refractory state to the antibody after the initial shock—all compatible with anaphylaxis—were demonstrated. The reacting substance was found to be in the gamma globulin fraction of the serum, and the antibody titer appeared to be correlated with the degree of shock and vascular injury.

Collagen injury in rats dying from acute shock or in those injected with repeated sublethal doses was not demonstrated by the methods employed; techniques by which lesions of collagen might be induced are discussed.

Absorption experiments indicated that anti-rat collagen rabbit serum is specifically directed to a substance, apparently collagen, in the rat; it is likely

that the combination of the antibody and collagen induces anaphylactic shock. These studies, made *in vivo*, provide further evidence of antigenic and immunological differences between acid-soluble rat and fish collagens.

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EXPLANATION OF PLATES

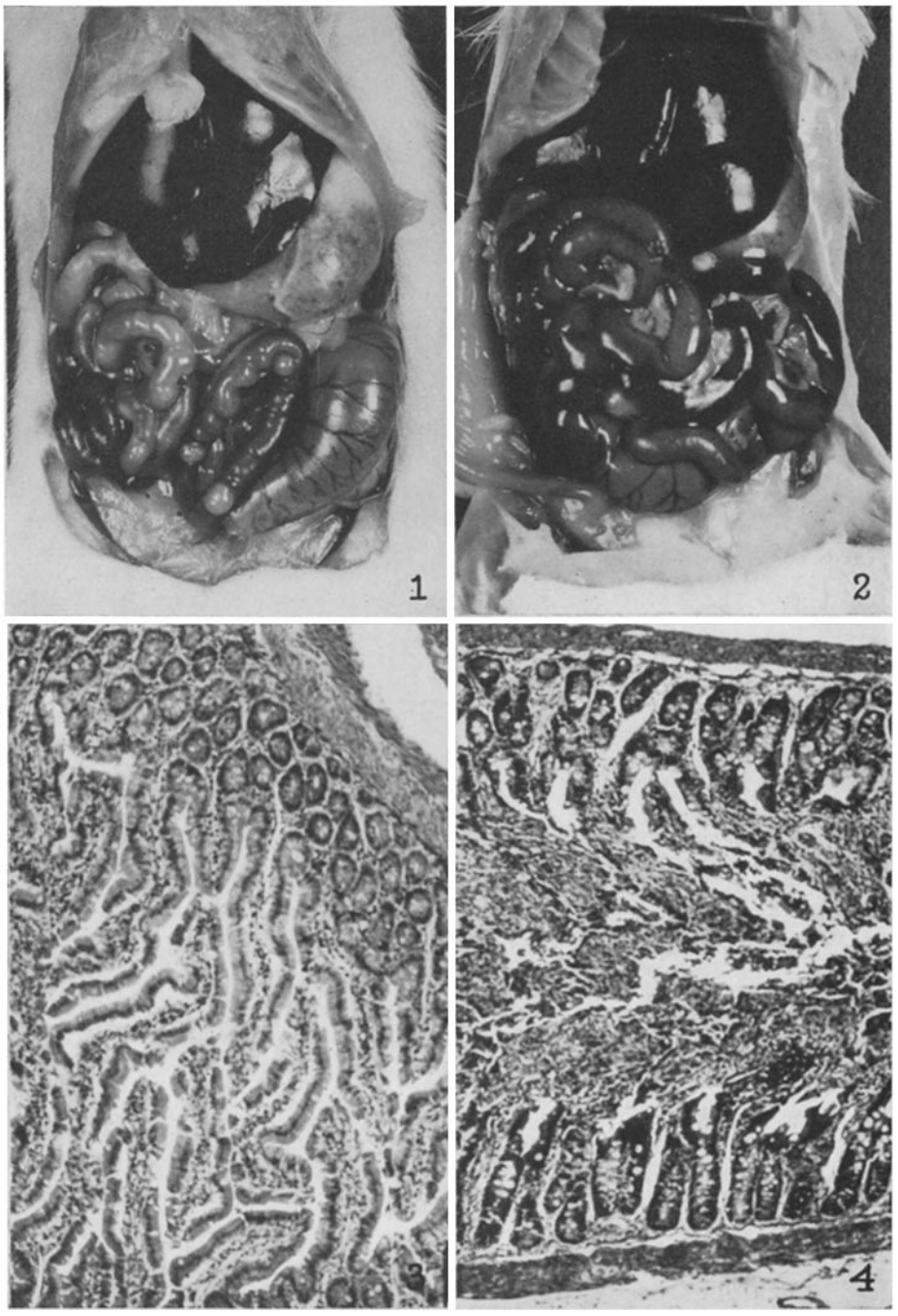
PLATE 2

FIG. 1. Abdominal viscera of control rat 6-3 sacrificed 30 minutes after intravenous injection with 5.0 cc. of normal rabbit serum. Natural size.

FIG. 2. Abdominal viscera of rat 6-4 which died from anaphylactic shock 30 minutes after intravenous injection of 5.0 cc. of anti-rat collagen rabbit serum, titer 1:128. Liver is enlarged and there is diffuse hemorrhage throughout the small intestine. Natural size.

FIG. 3. Same rat as in Fig. 1. Section of upper ilium showing normal structure. Hematoxylin and eosin stain, × 75.

FIG. 4. Same rat as in Fig. 2. Section of upper ilium showing necrosis of epithelial cells of intestinal villi and areas of hemorrhage. Hematoxylin and eosin stain, × 75.



(Rothbard and Watson: Antigenicity of rat collagen)

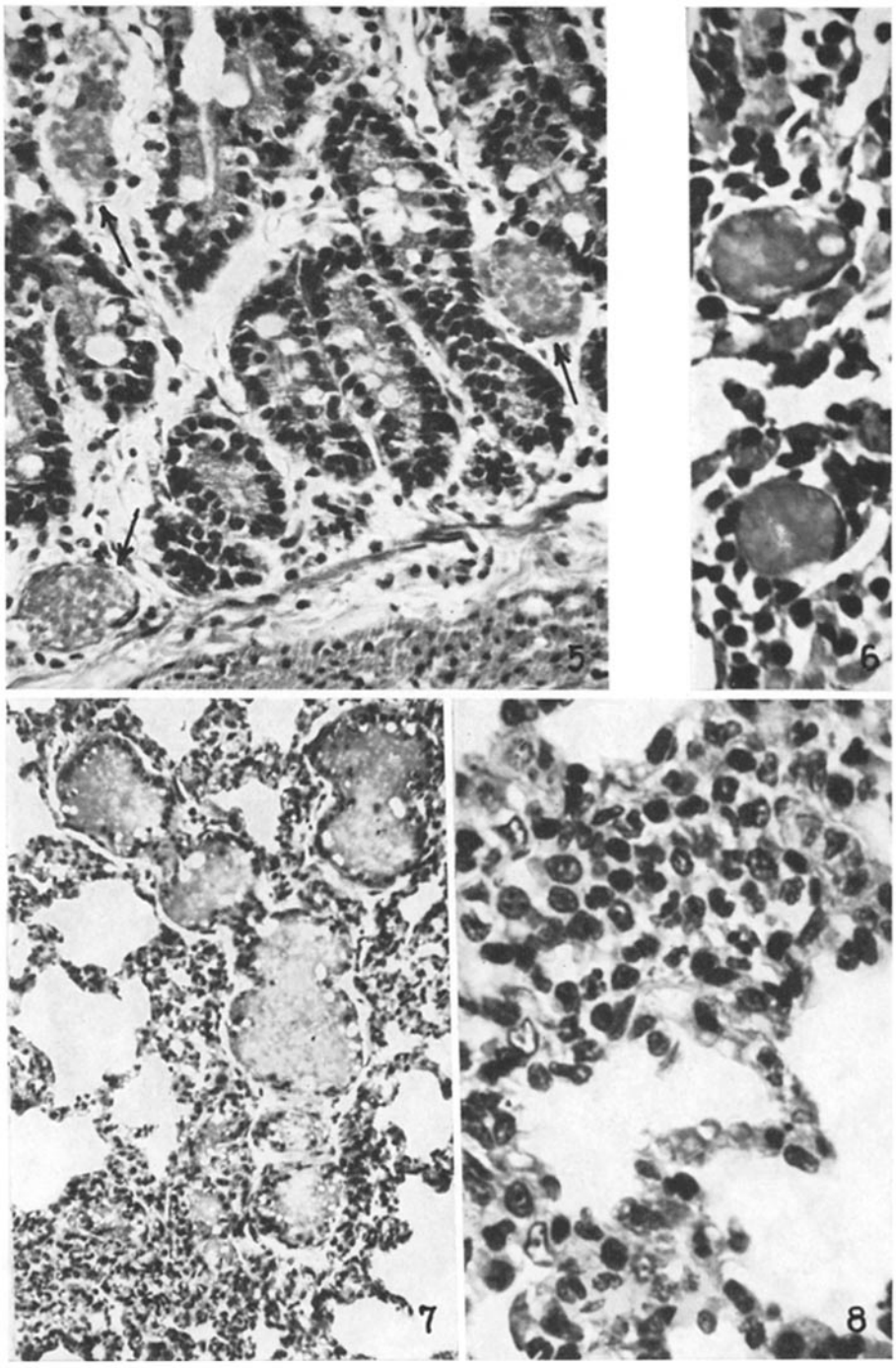
PLATE 3

FIG. 5. Higher magnification of Fig. 4. Arrows indicate erythrocyte thrombi in veins adjacent to the submucosa. Hematoxylin and eosin stain, × 360.

FIG. 6. Erythrocyte thrombi undergoing early hyalinization in veins of ilial mucosa of rat 9–3 which died from shock 90 minutes after injection of 4.0 cc. of anti-rat collagen rabbit serum, titer 1:128. Hematoxylin and eosin stain, × 580.

FIG. 7. Multiple erythrocyte thrombi in small veins and capillaries and thickened alveolar walls in lung of rat 9-2 which died from shock 90 minutes after injection of 4.0 cc. of anti-rat collagen rabbit serum, titer 1:128. Hematoxylin and eosin stain, × 380.

FIG. 8. Same rat as in Fig. 7. Sequestration of polymorphonuclear leukocytes in capillaries of thickened alveolar walls of lung. Hematoxylin and eosin stain, × 800.



(Rothbard and Watson: Antigenicity of rat collagen)