IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND.

I. Hemolysin Formation in Normal Rats.

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In the course of studies in this laboratory on the effect of suprarenalectomy on antibody formation, it became necessary to determine the hemolysin formation in normal rats. The hemolysin formation in several animals, particularly the rabbit, has been thoroughly investigated, but few studies on hemolysin formation in rats have been reported. In relation to a study of the effect of x-ray on antibody formation, Hektoen (1) produced fairly high hemolysin titers in rats with a small single intraperitoneal injection of sheep cells. Rats present certain peculiarities in immunity phenomena which make such studies of interest. A normal rat produces relatively slight precipitin (2). A state of local hypersensitiveness cannot be induced by injections of a foreign protein (2). Anaphylactic shock is produced with some difficulty (3). Rats withstand huge quantities of various poisons and toxins such as diphtheria (6), tetanus toxin (7) and histamine (4, 5).

Method.

The blood of the sheep was received in a preserving fluid recommended by Rous and Turner (8), consisting of isotonic solutions of glucose and sodium citrate. Cells were never kept longer than 6 days in this fluid and control daily fragility tests were employed. The cells used for injection and testing were washed three times with physiological salt solution to which was added .1 per cent of calcium chloride as recommended by Snapper (9) to prevent a disturbance of the osmotic equilibrium between the red blood cells and the saline solution. Single and multiple injections of varying quantities of antigen were given intraperitoneally and comparative studies of the hemolysin formation were made. Blood was obtained at intervals from the rats by puncturing the heart and withdrawing 1 to 2 cc. of blood. It was necessary to fix the rat on a board specially constructed and to

introduce the needle close to the sternum over the area of cardiac impulse. Repeated punctures at one sitting increase the dangers of fatal hemorrhage, but with practice, fatalities were reduced to less than 5 per cent.

In determining the titer of hemolysin present in the serum, progressive dilutions of the rat serum to be tested were made. .1 cc. of a 5 per cent suspension of sheep cells and 2½ units of complement were added. The total volume of each tube was brought up to 1 cc. with physiological saline. The tubes were incubated at 37° during 30 minutes and readings were made immediately. Traces of hemolysis equivalent to a one plus or lesser reading on a scale in which four plus represents complete hemolysis, were disregarded. That dilution in which hemolysis was partial but definite, that is, a two plus reaction, was read as the titer of the hemolysin present in the serum.

Hemolysin Formation after a Single Intraperitoneal Injection of Sheep Cells.

Twenty adult albino rats raised in the laboratory, from Wistar Institute stock, and all of approximately the same size and age, were used in this series. 1 cc. of a 10 per cent suspension of washed sheep cells was injected intraperitoneally into each rat. The titer was first determined 5 days after the injection and subsequent determinations were made on the 8th, 11th and 14th days. As noted in Table I, the height of the antibody formation is reached on or about the 5th day. The titer then gradually drops and is either very low or disappears entirely 14 days after the injection. The hemolysin titer in normal adult rats injected with 1 cc. of a 10 per cent suspension of sheep cells varies from 1:4000 to 1:24,000. Occasionally a titer of 1:2000 or 1:1600 is noted and in some rats the titer falls below this. The variation is considerable but it is not as striking as in rabbits. Although a high titer is reached with one injection of a small quantity of red blood cells, it is not maintained. The titer drops from 1:16,000 on the 5th day to 1:4000 or 1:2000 on the 8th day, and rapidly to 1:800 or 1:400 on the 11th day, while by the 14th day, it is about 1:200. The average initial titer of the twenty rats was 1:7000 on the 5th day; on the 8th day, the average titer was 1:3270; on the 11th, it was 1:580 and on the 14th day, the average titer was 1:200, although the titer in several animals had practically disappeared at this time.

A single injection of ten times this amount or 1 cc. of undiluted red blood cells was given to eight rats. It is seen from Table II that in these instances no titer exceeded 1:4000 and many titers were much

lower. The average initial titer in the rats in our series was 1:2000 after 5 days as compared with the average titer of 1:7000 of the previous series. The second and third readings were correspondingly lower (see Figs. 1 and 2).

Single injections of .5 cc. of a 10 per cent suspension had the same effect on hemolysin formation as 1 cc. of a 10 per cent suspension.

TABLE I.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Washed Sheep Cells and Tested at Intervals of 5, 8, 11 and 14 Days after the Injection.

Rat No.	Titer			
	5th day	8th day	11th day	14th day
61	6,000	2,000	600	100
62	2,000	200	160	200
63	24,000	8,000	1,600	600
64	2,000	2,000	200	80
65	32,000	16,000	2,000	600
66	3,000	2,000	160	150
27	4,000	Died		
30	300	160	10	20
88	8,000	800	1,000	300
89 A	12,000	1,000	800	100
103 A	1,600	Died		
104 B	400	1,000	80	
105	8,000	2,000	200	
134	1,600	1,600	600	
135	1,000	800	400	200
154	1,000	800	400	160
16	4,000	1,600	600	80
17	12,000	800	600	
23	16,000	2,000		
22	800	160		

However, with injections of .2 cc. of a 10 per cent suspension of sheep cells, antibody titers were considerably less than with 1 cc. of the dilution, although not in mathematical proportion. With single injections of .05 cc. of a 10 per cent suspension or one-twentieth the amount first used, the hemolysin titer rose to a maximum height of 1:800. As will be noted in Table III, the antibody formation in many

TABLE II.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of 1 Cc. of Undiluted Washed Sheep Cells and Tested at Intervals of 5, 8, 11 and 14 Days after the Injection.

Rat No.	Titer			
	5th day	8th day	11th day	14th day
139	4,000	600	400	30
140	4,000	2,000	800	80
141	1,600	400	160	
142	1,600	1,600	800	200
143	800	2,000	2,000	
144	1,600	200	400	
151	1,000	1,000	600	400
152	1,600	1,600		
153	2,000	600	—	400

TABLE III.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of Small Amounts of a 10 Per Cent Suspension of Washed Sheep Cells and Tested at Intervals of 5, 8 and 11 Days after the Injection.

Rat No.	Amount injected 10 per cent suspension	Titer		
		5th day	8th day	11th day
	cc.			
90	0.2	400	100	100
94	0.2	2,000	2,000	400
95	0.2	1,600	1,000	800
91	0.1	2,000	2,000	400
96	0.1	1,000	1,000	400
97	0.1	800	1,000	800
92	0.05	200	Died	
93	0.05	800	0	0
98	0.05	1,000	400	Died
136	0.05	400	400	30
137	0.05	400	400	80
138	0.05	200	400	160

instances was approximately one-twentieth as high as with twenty times the quantity of injected antigen. The relative curves with these varying amounts of antigen were similar, the titer disappearing on or about the 14th day (see Fig. 1).

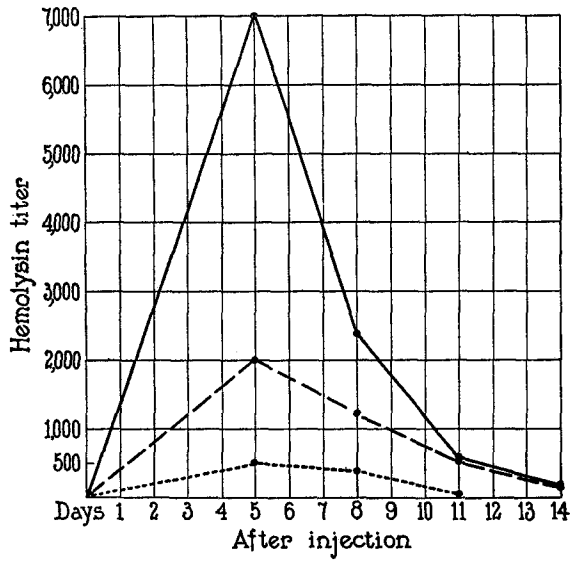

FIG. 1. Curves of average hemolysin titers with varying quantities of antigen.
——— Curve of average hemolysin titer of 20 normal rats injected with 1 cc. of a 10 per cent suspension of sheep cells.
— — — Curve of average hemolysin titer of 9 normal rats injected with 1 cc. of undiluted sheep cells.
-------- Curve of average hemolysin titer of 6 normal rats injected with 0.5 cc. of a 10 per cent suspension of sheep cells.

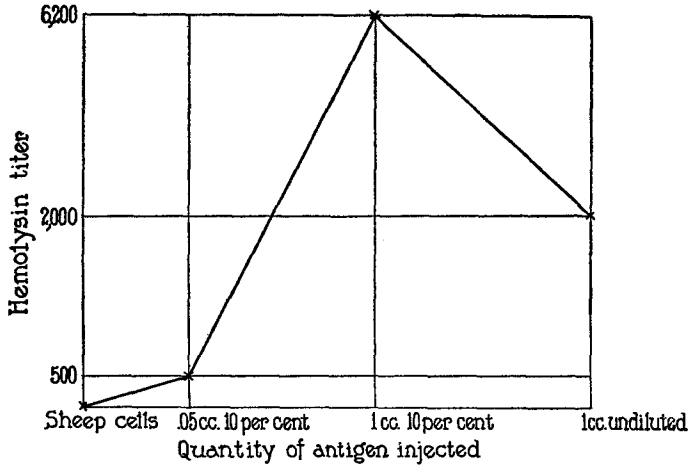

FIG. 2. Antigen-antibody curve in 35 normal rats injected intraperitoneally with varying quantities of sheep cells. The hemolysin titer in each instance is the average optimum titer obtained in each series.

From these experiments, it may be concluded that in normal adult albino rats, relatively high hemolysin titers may be obtained with a single intraperitoneal injection of a small quantity of sheep cells. There is, however, an optimum amount, 1 cc. of a 10 per cent suspension, which produces a definitely high titer. This titer rapidly drops and practically disappears from the blood after a period of 14 to 18 days. Amounts greater than this are not only ineffective in increasing the antibody formation but are followed by lower titers than those obtained with one-tenth the quantity of antigen. Much smaller

TABLE IV.

Hemolysin Formation in Normal Rats, Each Receiving Three Intraperitoneal Injections of Varying Amounts of Washed Sheep Cells at Varying Intervals and Tested 5, 8, 11 and 14 Days after the Last Injection.

Rat No.	No. of injections	Amount of each injection	Interval between injections	Titer			
				5th day	8th day	11th day	14th day
			days				
145	3	1 cc. 1/10	1	2,000	1,600	400	200
146	3	1 " 1/10	1	16,000	1,600	800	400
147	3	1 " 1/10	1	1,000	400	160	100
148	3	1 " undiluted	1	2,000	1,600	400	20
149	3	1 " "	1	1,000	400	30	40
150	3	1 " "	1	600	Died		
18	3	1 " 1/10	5	160	160	300	40
19	3	1 " 1/10	5	100	200	Died	
20	3	1 " 1/10	3	4,000	400	300	200
21	3	1 " 1/10	3	4,000	160	100	100

amounts produce correspondingly lower titers although the ratio is not always mathematically proportional.

Repeated Injections of Red Blood Cells.

Three injections each of 1 cc. of a 10 per cent suspension of sheep cells were given to three rats at daily intervals. The hemolysin titer was tested on the 5th, 8th, 11th and 14th days following the last injection. From Table IV it is seen that the antibody titer tested on the 5th day after the last injection is less than that resulting from a single injection of antigen. The first test was made on the 8th day following the *first* injection of antigen, and if Table IV is compared

with Table I, it is noted that the titer 5 days after the last of three injections repeated at daily intervals with 1 cc. of a 10 per cent suspension is about the same as on the 8th day following a single injection of 1 cc. of a 10 per cent suspension. It would seem that repeated injections at daily intervals failed to increase the antibody formation.

TABLE V.

Hemolysin Formation in Normal Rats, Each Receiving Three Intraperitoneal Injections of a 10 Per Cent Suspension of Washed Sheep Cells at 5 Day Intervals.

Rat No.	No. of injections	Date of injection	Amount injected 10 per cent suspension	Date tested	Titer
			cc.		
1	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	8,000
		9/17/27	0.5	9/17/27	200
				9/22/27	10
				9/26/27	0
2	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	10,000
		9/17/27	0.5	9/17/27	200
				9/22/27	0
3	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	16,000
		9/17/27	1.0	9/17/27	200
				9/22/27	40
				9/26/27	10
				10/ 1/27	20
4	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	10,000
		9/17/27	1.0	9/17/27	2,000
				9/19/27	200
				9/22/27	20
				9/26/27	10

*Sera tested before first injection contained no hemolysin.

Three injections at intervals of 3 days, each of 1 cc. of a 10 per cent suspension of red blood cells were given to two rats. The hemolysin titer was determined on the 3rd, 6th, 9th and 12th days following the last injection. Here again, it is noted, from Table IV, that the antibody titer was only slightly less than with a single injection.

Repeated injections at 5 day intervals, each of .5 cc. of a 10 per cent suspension for three injections, were given to several rats. The antibody titer was determined before each injection. From Table V it is seen that in spite of repeated injections of .5 cc. of a 10 per cent suspension of red blood cells, the titer never exceeded the initial rise which it reached 5 days after the first injection. Furthermore, in spite of repeated injections, the titer dropped progressively exactly as in the instances with a single injection. Subsequent injections had practically no effect on the curve of antibody formation produced by the first injection.

Repeated injections of similar amounts of antigen at daily intervals, or at intervals of 3 or 5 days, do not increase the antibody titer nor do repeated subsequent injections of the same amount of antigen modify the curve of hemolysin formation resulting from the first injection.

DISCUSSION.

The curve of the hemolysin titer in healthy rabbits has been carefully studied by Bulloch (10), Sachs (11), Lüdke (12) and Remy (13). Following single or multiple injections of sheep cells, Bessau and Paetsch (14) and Sachs (11) observed a negative phase during which no antibodies were detected in the serum. The length of this negative phase was observed by Sachs to be entirely independent of the amount of blood cells injected, but varied with the method of injection. Following the subcutaneous route, this period was generally 7 days, but in instances in which the intravenous or intraperitoneal route was used, this latent period was reduced to 3 days (Bulloch) or to 2 to 4 days (Sachs). The hemolysin titer then rose to a maximum on the 5th to the 6th day where it was maintained for 24 hours after which it gradually fell. Wolf (15) observed a second rise in rabbits on the 7th to the 10th day. The titer then gradually dropped. There was no direct proportion between the amount of antigen injected and the antibody titer which was produced. Sachs studied the relation of amboceptor to red blood cells and complement in rabbits and found that the appearance of the immune body coincided with the disappearance of the foreign red blood cells from the circulation of the immune animal. Lüdke (16) found that ox cells injected into rabbits subcutaneously completely disappeared in 8 days from the circulation, when hemolysin could first be detected. Probably the immune substances first formed by the injection of the antigen hemolyzed the red blood cells present in the circulation by specific action. In this process complement was also utilized. With the neutralization of the antigen present, the amboceptor became detectable.

In our work we have found that repeated injections of small amounts of red blood cells did not increase the titer of the hemolysin formed by a single injection nor did subsequent injections maintain the titer of hemolysin produced by the first injection. Possibly the amounts injected after the first injection were hemolyzed by existing amboceptor in the serum of the immunized animal and were therefore unable to stimulate further antibody production.

Coca (17) found that after a single injection of 1 cc. of a 10 per cent suspension of sheep cells in rabbits, average titers 1 week after injection reached a maximum of 1:250 (.004). When twenty times this quantity was used, the titer obtained was 1:154 (.0065), definitely lower than with the larger amounts. In rats, we found a similar antibody-antigen relation; with ten times the quantity of antigen, the amboceptor titer dropped. Injections of smaller quantities of antigen in rabbits, Coca found, were more effective in producing a high titer than injections of larger amounts. The observations of Coca and others have shown, however, that in rabbits the number of injections and the intervals between injections influence the hemolysin titer. In rats, the titer following the initial injection of sheep cells was not raised by repeated injections of the same amount of sheep cells. Further subsequent injections had little effect on the curve of the hemolysin formation initiated by the first injection.

SUMMARY AND CONCLUSION.

Hemolysin formation was studied in normal rats. It was found that a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of red blood cells results in an optimum high hemolysin titer 5 days after injection. The titer gradually falls, the hemolysin disappearing from 14 to 18 days after the injection. Larger amounts are less effective in the production of amboceptor. Much smaller amounts produce correspondingly lower hemolysin titers, although the ratio is not mathematically proportional. Repeated injections of 1 cc. of a 10 per cent suspension of red blood cells at daily intervals or at intervals of 3 or 5 days for three injections do not increase the hemolysin titer over that resulting from a single injection. Furthermore, the curve of antibody formation following a single small intraperitoneal injection of red blood cells is not altered by subsequent injections of similar amounts.

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