THE LIVER AS A SOURCE OF BACTERIAL AGGLUTININ.

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The locus of antibody formation has not been definitely determined. The opinion at first advanced by Metchnikoff that antibodies originated in the leucocytes failed of experimental proof. During the past few years particular attention has centered about the spleen, lymph nodes, and bone marrow as important organs of hemolysin formation. Hektoen[1] showed that splenectomized rats developed hemolysin to a less degree than the normal controls. This did not hold true for the rabbit. Kyes[2] called attention to the fact that foreign red cells introduced into the circulation are destroyed by the endothelial phagocytes of the liver and spleen. Cary,[3] impressed by Kyes' observations, developed the hypothesis, from a series of experiments, that the splenic endothelial cells which phagocyted the foreign red cells produced the hemolysin. He based his conclusions on evidence that hemolysin was much more concentrated in the spleen than in the liver or serum of immunized rabbits. Motohashi[4] showed that the physiological hemophagic activity in the rabbit is confined to the spleen. After splenectomy, however, there occurs after a certain period, a compensatory hemophagic activity in the bone marrow and liver. He was able to show that rabbits injected 2 or 3 weeks after splenectomy, or during the period when the compensatory hemophagic activity was at a minimum, developed hemolysin to a much less degree than the controls.

Experiments dealing with the formation of bacterial antibodies are on the whole less convincing. Wassermann and Citron[5] determined that lysin was more concentrated in the liquid portions of the exudates produced by intraperitoneal or intrapleural injection of typhoid bacilli than it was in the blood serum. It was suggested that the lysin was made by the cells of the cavities. Hektoen[6] was unable to confirm this in the case of dogs injected with foreign

[1] Hektoen, L., *J. Infect. Dis.*, 1920, xxvii, 23.

[2] Kyes, P., *Internat. Monatschr. Anat. u. Physiol.*, 1914–15, xxxi, 543.

[3] Cary, W. E., *J. Med. Research*, 1922, xliii, 399.

[4] Motohashi, S., *J. Med. Research*, 1922, xliii, 419; 473.

[5] Wassermann, A., and Citron, J., *Z. Hyg. u. Infectionskrankh.*, 1905, i, 331.

[6] Hektoen, L., *J. Infect. Dis.*, 1911, ix, 103.

red cells. Pfeiffer and Marx,[7] Wassermann,[8] and Castellani[9] all showed that bacteriolytic substances were more concentrated in the spleen, bone marrow, and lymph nodes early in immunization than they were in the blood serum.

Isolated experiments, however, indicate that antibody may be produced by other tissues. One of Wassermann and Citron's observations suggests that the tissues surrounding the site of inoculation may produce antibody to a certain extent. Zinsser[10] cites the findings of Römer[11] who instilled abrin into a rabbit's eye and found that the retina developed strong antitoxic properties. The retina of the untreated eye remained inactive. Theobald Smith, Orcutt, and Little[12] have shown that the milk drawn from a quarter infected with *B. abortus* contains agglutinin in higher concentration than milk from uninfected quarters. As a result of a series of experiments in which various quarters were inoculated with living or dead cultures of *B. abortus* they concluded that there is a distinct participation of the udder in the production of agglutinin. Recently Murphy and Sturm[13] have shown that there is an apparent relationship of the lymphoid structure to the production of antibody. They showed that x-rays in doses sufficient to deplete lymphoid tissues seriously interfered with the production of precipitin and agglutinin. Dry heat, shown by them to stimulate the activity of lymphoid structures, leads to the production of antibody in greater concentration.

Inasmuch as most of the previous studies dealt with antibody other than agglutinin it seemed desirable to study other possible points of origin than those already mentioned. A procedure which did not affect the general health of the animal or alter the normal metabolic processes was particularly desirable. With these points in view the following methods were adopted.

Methods.

It seemed probable that extracts could be prepared from dried material. With such a procedure more accurate comparisons would be possible, since tissues rich in liquids could be compared with those of low water content on the basis of total dry matter. It will be shown

[7] Pfeiffer, R., and Marx, *Z. Hyg. u. Infectionskrankh.*, 1898, xxvii, 272.
[8] Wassermann, A., *Berl. klin. Woch.*, 1898, xxxv, 209.
[9] Castellani, A., *Z. Hyg. u. Infectionskrankh.*, 1901, xxxvii, 381.
[10] Zinsser, H., Infection and resistance, New York, 3rd edition, 1923.
[11] Römer, cited by Zinsser.[10]
[12] Smith, T., Orcutt, M. L., and Little, R. B., *J. Exp. Med.*, 1923, xxxvii, 153.
[13] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1925, xli, 245.

that desiccated material when extracted with distilled water admirably meets these requirements.

The vaccine employed was of standard turbidity. It was prepared by suspending the growth from 24 hour slant agar cultures of the hog cholera bacillus in salt solution. The suspension was heated at 60°C. for 1 hour.

The rabbits, of approximately the same age and weight, were injected intravenously. After varying intervals they were stunned and the neck vessels severed, according to the procedure used in an abattoir. In this way the tissues were freed from considerable blood. As rapidly as possible the organs were removed and ground in mortars. The tissues were then spread in small Petri dishes in thin layers and dried *in vacuo* over sulfuric acid. All tissues were completely desiccated within 24 hours. Structures rich in lipoids, like the bone marrow and lymph nodes, remained greasy, often of the consistency of butter. The desiccated material was ground into powder and stored in tightly stoppered tubes. For use it was weighed and suspended in distilled water in the proportion of 0.2 gm. of powder to 4 cc. of water. This proportion was finally chosen as a standard after several trials. It was found that this amount of dried rabbit serum would be dissolved in 4 cc. of distilled water. Extraction was carried out in tightly stoppered tubes for 4 hours at 36°C. All tubes were shaken at 15 minute intervals. They were then stored in the refrigerator for 16 or 18 hours and again shaken and centrifuged. The supernatant liquid varied in color and opacity. It was tested for agglutinin content against salt solution suspensions of living antigen.

The process of desiccation and extraction described might conceivably injure the antibody or so change it as to render it insoluble. To test this point the following experiment was performed.

Experiment 1.—3 cc. of the serum of an immunized rabbit, rich in agglutinin for the hog cholera bacillus, was dried over sulfuric acid *in vacuo*. 0.202 gm. of the dried material was obtained. 0.2 gm. of this was extracted with 4 cc. of distilled water in the manner described. As a control, 1 cc. of distilled water was added to 3 cc. of the immune serum. Both were tested for agglutinin content with a suspension of living hog cholera bacilli. The results of the comparative tests are given in Table I.

It will be noted that the agglutinin is not quite as strong in the extract of dried serum as in the serum unchanged except for the addition of distilled water. The experiment, however, indicates that the antibodies were not seriously injured during the process of desiccation. It is further indicated that the extraction of the dried serum with distilled water is efficacious. The method then was sufficiently

TABLE I.

A Comparison of the Agglutinin Titer of Dried Immune Serum and That of Serum Diluted to the Same Concentration with Distilled Water.

Materials tested.	Dilutions.						
	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000	1:10,000
Extract of dried serum.....	C.*	C.	C.	++++	+++	++	++
Control serum diluted to 1:3 with distilled water.....	C.	C.	C.	C.	+++	+++	+++

* Throughout the tables, C. indicates a complete agglutination; the degree of precipitation is given in terms of +: ++++ indicates a strong agglutination, and the weaker are recorded as +++, ++, +.

TABLE II.

Native Agglutinins for the Hog Cholera Bacillus in the Extracts Prepared from Blood, Serum, and Organs of Normal Rabbits.

Rabbit No.	Extracts tested.	Dilutions of extracts.				
		1:5	1:10	1:20	1:50	1:100
1	Blood.	+	±	−	−	−
2	"					
1	Serum.	++	+	+	−	−
2	"	++	+	−	−	−
1	Lung.	±	−	−	−	−
2	"	±	−	−	−	−
1	Liver.	++	+	−	−	−
2	"	++	+	−	−	−
1	Spleen.	±	−	−	−	−
2	"	±	−	−	−	−
1	Kidney.	±	−	−	−	−
2	"	−	−	−	−	−
1	Lymph glands.	±	−	−	−	−
2	" "	+	−	−	−	−
1	Bone marrow.	−	−	−	−	−
2	" "	−	−	−	−	−

accurate for further experimentation. In the experiments the extracts were prepared usually within a week. The material was retested again after storage for 2 weeks at room temperature. A few tests made on material stored at room temperature over the summer showed that the serum, liver, and other organs were no longer as soluble as when freshly dried.

The next step was to establish the agglutinin content of various organs of normal animals.

Experiment 2.—On different occasions normal rabbits were killed and bled in the manner described. Portions of blood, serum, lungs, liver, spleen, kidneys, lymph glands, and bone marrow were dried *in vacuo* over sulfuric acid. Aqueous extracts were prepared and tested for natural agglutinin with salt solution suspensions of the hog cholera bacillus. It was found that the excess of hemoglobin of whole blood prevented accurate readings and its use was therefore discontinued. The results obtained from two normal rabbits are given in Table II.

The results of a number of examinations of normal animals have been that the natural agglutinin is always present in the serum at a slightly higher concentration than in the organs. Of the organs the liver is richest in antibody, but in the normal individual its concentration is slight.

Having established the normal agglutinin content of the various organs the next step undertaken was to ascertain if possible in what organs the greatest concentration of agglutinin occurred during immunization. At first I attempted to establish this by intravenous injections of several small doses of vaccine, permitting several days to elapse between the last injection and examination of the tissues. It was found, however, that with such a procedure the antibody was more concentrated in the serum than in the tissues. The details of a single observation illustrating this fact are given in Experiment 3.

Experiment 3.—Rabbit 3 was injected intravenously on 3 successive days with six small doses of vaccine. The reactions following each injection were severe. The amount of agglutinin in the blood was followed from day to day. 5 days after the last injection the animal was killed. The serum and organ extracts were prepared in the usual way. The results are given in Table III.

In the highly immunized animal the antibody is at its greatest concentration in the blood serum, as the table indicates. Hektoen and

Carlson,[14] however, have clearly shown that the blood itself is incapable of producing antibody. Therefore it is possible that the period between the last injection of antigen and the tests of the organs was of sufficient length to permit the passage into the blood stream of the major portion of antibody from the site of its formation. The results of the tests of certain organs, however, were suggestive. The liver and kidneys possessed the greatest concentration of antibody; there was less in the spleen and lymph glands. With these facts established it was decided to continue immunization along the same lines but to inject smaller doses of vaccine and to decrease the interval between the last injection and the death of the animal.

TABLE III.

The Agglutinin Content of the Extracts of Dried Serum and Organs of a Rabbit Immunized by Multiple Doses of Vaccine.

Extracts tested.	Dilutions of extracts.							
	1:5	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000
Serum	C.	C.	C.	+++	++	++	+	±
Lung	++	+	+	±	−	−	−	−
Liver	C.	+++	++	+	+	+	+	±
Spleen	+++	++	++	+	+	±	−	−
Kidney	C.	+++	+	+	+	+	±	−
Lymph gland	C.	++	+	+	+	±	−	−
Bone marrow	++	++	+	+	±	−	−	−
Testicle	+	+	±	−	−	−	−	−
Muscle	+++	+	±	−	−	−	−	−
Small intestine	+++	+	+	±	−	−	−	−

The following may be regarded as a type experiment. The protocols of two rabbits only are submitted. Other rabbits treated in the same manner gave similar results.

Experiment 4.—Rabbit 4. Bled before injection. Its serum when tested only slightly clumped the bacilli at a dilution of 1:5. The animal in the course of 2 days received three intravenous injections aggregating 0.85 cc. of vaccine. Moderate temperature reactions followed each injection. 25 hours after the last injection the animal was killed.

[14] Hektoen, L., and Carlson, A. J., *J. Infect. Dis.*, 1910, vii, 319.

Rabbit 5. The blood serum obtained before injection failed to agglutinate the antigen at a dilution of 1:5. Four intravenous injections of vaccine aggregating 1.85 cc. were made during 2 days. The animal was killed 48 hours after the last injection.

The results of the tests are recorded in Table IV.

TABLE IV.

The Agglutinin Content of the Dried Serum and Organs of Rabbits Immunized by Intravenous Injections and Killed 1 and 2 Days after the Last Injection.

Rabbit No.	Extracts tested.	Dilutions of extracts.					
		1:5	1:10	1:20	1:50	1:100	1:200
4	Serum.	++	+	−	−	−	−
5	"	++	+	±	±	−	−
4	Lung.	++	+	+	±	−	−
5	"	+++	++	+	±	−	−
4	Liver.	++++	+++	++	+	±	±
5	"	++++	++++	+++	++	+	±
4	Spleen.	+	±	±	−	−	−
5	"	+++	++	+	±	−	−
4	Kidney.	+++	++	+	±	−	−
5	"	+++	++	+	±	−	−
4	Lymph gland.	+	−	−	−	−	−
5	" "	++	+	+	±	−	−
4	Bone marrow.	+	±	−	−	−	−
5	" "	+	+	±	±	−	−

The results of the tests indicate that early in the period of immunization agglutinin is present in greater concentration in certain organs than it is in the serum. The liver contains agglutinin in the greatest concentration under these conditions. The lungs and kidneys contain, as a rule, more than the spleen, lymph glands, and bone marrow. The inference then that antibody is produced to a considerable extent in several organs, especially the liver, seems reasonable. Several explanations of the high concentration of antibodies in the liver must be considered. Considerable blood may have remained in the organ

but only 50 or 60 per cent of this could have been serum, since the titer of the serum is considerably below that of the liver extract. Another point that might be raised is the possibility of the liver as a storage place for antibodies. The results given in Table III indicate, however, that when the serum of a highly immunized individual is rich in antibody the liver contains considerably less. It seemed possible to definitely settle this question by the following experiment.

Experiment 5.—Rabbit 6. Injected intravenously with 4 cc. of immune rabbit serum. 1/2,000 cc. of the recipient's serum obtained shortly after the injection would clump the bacilli. The animal was bled at daily intervals. At the end of 7 days the concentration of agglutinin in the circulating blood had markedly declined and the animal was killed. Portions of the serum and organs were desiccated and extracted. The extracts when tested for agglutinin gave the results recorded in Table V.

TABLE V.

The Concentration of Agglutinin in the Serum and Tissues of a Passively Immunized Rabbit.

Extracts tested.	Dilutions of extracts.					
	1:10	1:20	1:50	1:100	1:200	1:500
Serum	C.	+++	++	+	±	−
Lungs	++++	++	±	−	−	−
Liver	++	+	+	−	−	−
Spleen	+	±	−	−	−	−
Kidneys	+	+	±	−	−	−
Lymph gland	+	+	±	−	−	−
Bone marrow	±	±	−	−	−	−
Muscle	+	±	−	−	−	−

Similar results were obtained when another rabbit was treated in the same manner. This experiment amplifies Experiment 2 and indicates that the liver is not a storage place for agglutinin since it persists in the blood in greater concentration than in any of the organs. It has long been known that in passive immunity antibodies disappear from the blood. The assumption has been that they are eliminated from the body. This explanation would seem to be the correct one, since they are not stored in the organs.

A final series of experiments seemed desirable. Evans, Bowman,

and Winternitz[15] showed that dead tubercle bacilli injected into a radicle of the mesenteric vein were transported to the liver and ingested by Kupffer cells. By the use of a similar technique it was hoped that most of the antigen would first come in contact with the liver and there be utilized, thus giving rise to a greater concentration of agglutinin within the organ. While this proved to be not wholly the case the results indicate that in the main it was true. An outline of the procedure and findings is given in the following experiment.

Experiment 6.—Rabbit 7. Anesthetized with ether. A median line incision was made through the skin and tissues which comprise the abdominal wall.

TABLE VI.

The Concentration of Agglutinin in the Serum and Organs Subsequent to Injection of Antigen into a Radicle of the Mesenteric Vein.

Extracts tested.	Dilutions of extracts.					
	1:5	1:10	1:20	1:50	1:100	1;200
Serum	+	±	−	−	−	−
Lungs	−	−	−	−	−	−
Liver	C.	C.	+++	+	+	±
Spleen	+	+	±	−	−	−
Kidneys	+	+	±	−	−	−
Ascitic fluid	+	+	±	−	−	−
Bone marrow	±	−	−	−	−	−
Small intestine*	+++	+	−	−	−	−
Lymph gland	+	±	−	−	−	−

* Includes the mesentery surrounding the site of injection.

With rubber covered forceps a loop of small intestine was withdrawn through the opening and a fine hypodermic needle introduced into a branch of the mesenteric vein and 0.75 cc. of standard vaccine together with 1.25 cc. of salt solution slowly injected. The incision was closed with silk and a suspensory bandage applied. The animal made an uneventful recovery. 4 days later it was killed and the serum and tissues desiccated. Autopsy failed to show gross changes in the viscera. There was a little clear fluid in the abdominal cavity. The wound was uninfected and healing processes well advanced. The results of tests of the extracts are shown in Table VI.

[15] Evans, H. M., Bowman, F. B., and Winternitz, M. C., *J. Exp. Med.*, 1914, xix, 283.

Another rabbit killed 3 days after a smaller injection into a branch of the mesenteric vein also developed a concentration of agglutinin in the liver ten times as great as that of the blood serum and five times greater than in the spleen. In these instances presumably most of the antigen first came in contact with the liver. It seems probable that it was utilized within this organ.

A number of other experiments were performed in which rabbits were injected intratracheally, intramuscularly, and intratesticularly. In certain instances the concentration of agglutinin was slightly greater in the liver than in the other organs and serum, but the results were not as definite as those reported when the injections were made intravenously.

The method of drying and extraction seemed well adapted to the study of the local formation of antibodies. For the purpose rabbits were injected intramuscularly and intratesticularly with vaccine, and in certain instances the site of injection was first prepared by the administration of kieselguhr or agar. In no instance was it possible to show that the injected muscle or testicle possessed more antibody than the corresponding uninjected areas.

DISCUSSION.

Of considerable interest are the findings of others concerning the destruction of bacteria introduced into the blood stream.

Werigo[16] pointed out that anthrax bacilli injected intravenously were disposed of by the hepatic endothelium. Adami, Abbott, and Nicholson[17] injected colon bacilli into the veins of young rabbits and killed them at intervals. They noted that within a short time the endothelial cells lining the capillaries of the liver began to phagocyte the bacilli. Within 2 hours the endothelial cells contained large numbers of bacilli and no free bacilli remained in the capillaries. They pointed out that certain cells of the kidney possessed the same faculty but to a less degree. Evans, Bowman, and Winternitz, whose experiments have been previously referred to, were able to show that dried tubercle bacilli when they reached the liver were taken up by Kupffer cells. Kyes[18] in studying the natural resistance of the pigeon to the pneumococcus encountered the same phenom-

[16] Werigo, *Ann. Inst. Pasteur*, 1894, viii, 1.

[17] Adami, J. G., Abbott, M. E., and Nicholson, F. J., *J. Exp. Med.*, 1899, iv, 349.

[18] Kyes, P., *J. Infect. Dis.*, 1916, xviii, 277.

enon. He pointed out that the cocci were rapidly taken from the liver circulation by the hemophages lining the capillaries. He also stated that the hemophages from the spleen contained as many cocci cell for cell as those of the liver, but in the spleen such cells are much less numerous than in the liver. Certain specific peculiarities of the anatomical structure of the walls of the smaller splenic vessels of the pigeon permit the intravascular accumulation of pneumococci, greatly facilitating phagocytosis. Bull[19] noted that typhoid bacilli were destroyed by phagocytes in the liver and spleen of rabbits which had received large doses of culture intravenously. He considered the polymorphonuclear leucocytes of prime importance in the process.

It will be seen that there is considerable accord concerning the importance of the liver as a locus of accumulation and destruction of bacteria entering the blood stream. With the small doses of dead bacteria employed in my experiments it seems entirely possible that a great bulk of the bacteria were destroyed by the liver and that the agglutinin was formed there during the process. The importance of the endothelial cells lining the capillaries as active phagocytes of bacteria and other substances suggests these cells as a possible source of antibody formation. The fact that the "plugging" of endothelial phagocytes with trypan blue inhibits antibody production, as recorded by Gay and Clark,[20] strengthens such a view.

It is usually conceded that antigens injected intravenously lead to a more rapid and usually to a greater production of antibodies than injections by other routes. It is also true that the reactions occurring from such a procedure are usually more severe. Under such conditions much of the antigen, particularly bacterial cells, may accumulate in the liver where there exists ample opportunity for rapid phagocytosis and consequent destruction. Antigen injected by routes other than the blood stream would reach the liver and other organs more slowly and the resulting reactions would be milder in character and perhaps lead to a slower increase in antibodies. It should be emphasized that in the opinion of the writer the liver is not the only organ of importance in agglutinin formation. Doubtless agglutinins are formed in many structures. The work of Theobald Smith, Orcutt, and Little indicates that the udder plays a part in

[19] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 475.

[20] Gay, F. P., and Clark, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 1.

agglutinin production. The experiments indicate, however, that bacterial antigen introduced into the systemic or portal circulation leads to a considerable increase of antibody within the liver.

SUMMARY.

Serum and tissues containing agglutinin for the hog cholera bacillus may be dried *in vacuo* over sulfuric acid without appreciably injuring the antibody. The desiccated material when extracted with appropriate amounts of distilled water offers a basis for accurate comparison of antibody content. The greatest concentration of agglutinin occurred in the liver, provided the animals injected with small amounts of antigen were killed within a short period. The serum of those more highly immunized contained the greatest concentration of antibody. A single injection of antigen into a radicle of the mesenteric vein resulted in a considerable concentration of agglutinin in the liver. Other experiments indicated that the liver does not act as a reservoir for the antibody. It has also been shown that this concentration of agglutinin cannot be ascribed to the blood left within the liver, since the blood serum was relatively poor in antibody. The experiments indicate that the agglutinin was produced within the liver.