THE PATHOGENESIS OF EXPERIMENTAL DYSENTERY INTOXICATION

PRODUCTION OF THE LESIONS BY CEREBRAL CIRCULATION OF THE TOXIN*

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In the course of studying the mechanism whereby Shiga toxin produces visceral lesions, it became increasingly clear that the toxin produced its effects indirectly. Direct contact between the toxin and intestinal mucosa in dogs produced no lesions in the exposed loop (1). Certain anatomical peculiarities of the lesions pointed to a vascular component in the development of the structural changes. It was found that the widely distributed alterations usually associated with the morphological picture of Shiga enteritis occurred in a late, and often terminal, stage of the intoxication. Serial studies timed to investigate the early stages of the lesions revealed that the initial changes were focal in nature, and that the later alterations were the result of the confluence of numerous focal lesions (1). There were no visible alterations in the structure of the vessel walls, but the tissue edema and extravasation of blood cells left no doubt as to changes in permeability of the vascular bed. These "functional" alterations were attributed to the occurrence of vasospasm in the involved areas (2). Subsequent studies were devoted to an analysis of the mechanism of the production of the vasospasm. We were able to show that this occurred in close temporal relationship to the appearance of hemoconcentration and hyperglycemia. As a result, we hypothesized that the vasospasm resulted from a homeostatic reaction on the part of the organism. This diffuse, systemic sympathomimetic reaction produced the hyperglycemia on the one hand and on the other caused the vasospasm. The hemoconcentration which was uniformly found was interpreted as the result of the increased permeability of the capillaries which simultaneously produced the edematous and ulcerative lesions in the alimentary tract.

In further studies it was found that by pretreating our animals with ergotamine tartrate we were able to inhibit the hyperglycemia, hemoconcentration, and occurrence of the anatomical lesions (3). This indicated that the point of primary action of the toxin was somewhere proximal to the final common

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pathway of the autonomic nervous system. It did not permit us to decide whether this was in the adrenal medulla or in the central nervous system or both. Further investigation, using tetraethyl ammonium chloride, showed that this substance also inhibited the phenomena (4) mentioned above. Since this drug acts by inhibiting the passage of autonomic impulses through sympathetic ganglia, these results were interpreted as evidence that Shiga toxin has its primary action in the central nervous system itself.

The present studies were designed to investigate this conception directly by means of cross-circulation experiments.

Methods

The toxin used was prepared by the "slow" method of Olitzky and Kligler from a strain of *Bacillus shigae* kindly supplied to us by Dr. Sarah Branham of the National Institutes of Health, Bethesda. The toxin was not standardized, but as a result of a series of trials, it was found that lesions were invariably produced in the small intestine and/or gall bladder when it was given intravenously in a dose of 1 cc. per kilo of body weight. In fifteen dogs in which this amount was given, typical lesions occurred in each instance.

Hematocrit determinations were made by the method of Wintrobe (5). The hematologic studies were done with standard pipettes and chambers. The same pipette and chamber were used in each count. The blood smears were stained with Wright's stain. The blood used for the hematologic determinations was treated with an anticoagulant. An aqueous solution of 6 per cent ammonium oxalate and 4 per cent potassium oxalate was used for this purpose. To 0.2 cc. of this solution 1.8 cc. of venous blood was added and shaken in a small vial. This was done even when the blood had been rendered incoagulable by the intravenous injection of heparin, in order to insure constant conditions for our counts.

In addition, two specimens of venous blood of 5 cc. each were procured. In three experiments one of them was collected under mineral oil, and both were kept under refrigeration until determinations of sugar, chloride, and carbon dioxide were made. Sodium fluoride crystals were added to the specimen in which the sugar was to be determined. Clotting was prevented by collecting the blood in tubes containing 0.25 mg. solid heparin. The chloride and carbon dioxide were determined by the methods of Van Slyke; the sugar by a modification of the Folin-Wu technique (6). We did not determine the blood groups of our animals (7) but the blood of each member of a pair was tested by cross-agglutination in test tubes with its partner, in order to assure compatibility.

Procedure

The data reported here are the results obtained in cross-circulation experiments designed to study the effects of Shiga toxin in dogs.

Six pairs of dogs were used, each surgically prepared in stages so that, during the final experiments, the circulation of the brain of each dog, while isolated from its own systemic circulation, was supplied completely by the visceral circulation of the other dog of the pair.

This cross-circulatory arrangement was accomplished as follows:—

Preliminary ligation and division of the vertebral artery and vein were performed on each side of the neck in the vertebral canal of the transverse process of the sixth cervical vertebra. The operations were performed in two stages at intervals of several weeks, each side being done separately in order to avoid paralysis of the hind legs. This complication had been noted occasionally in preliminary experiments when the vertebral vessels were cut bilaterally at one operative sitting.

In the dog, the vertebral artery divides into three branches between the second and third cervical veterbrae. Two of these form anastomoses which contribute to the blood supply of the brain; the third supplies portions of the cervical musculature. The level of the sixth cervical vertebra was therefore selected in order to secure complete interruption of the cerebral blood flow *via* the vertebral vessels, and to avoid systemic anastomotic circulation (8).

Using intravenous nembutal anesthesia, the animal was placed on its side with the neck arched laterally over a firm support, and a cervical incision was made obliquely downward from the second transverse process, following a line joining the lateral tips of the transverse process to beyond the sixth cervical vertebra. Employing careful hemostasis throughout, musculofascial planes were separated to expose the fifth transverse process. This was then resected subperiosteally in order to provide better access to the sixth transverse process, which partially underlies it. The latter was next cleaned off subperiosteally, permitting it to be completely exposed down to its origin in the vertebral body by retracting the musculature which had been freed by this maneuver. The transverse process was cut away with a rongeur, almost to its base. By curetting the superior wall of the transverse process in this basilar area, using Richards' mastoid curettes, the bone was thinned sufficiently to permit visual identification of the vertebral vessels in their canal. The superior and lateral walls of the vertebral canal were further thinned by curette and the remaining thin bone could now easily be cut away with a fine, sharp Lempert rongeur. The vertebral vessels were freed with a blunt instrument from their periosteal attachment to the canal walls, and silk ligatures were passed about the vessels at each end of the canal with a very fine ligature carrier. These ligatures were doubly tied, and the vessels cut across between them. During these procedures care had to be taken to avoid severing a small branch vessel which enters a foramen in the anterior wall of the canal. Serious, sometimes fatal hemorrhage occurred if it was opened before the vertebral vessels themselves had been ligated above and below it.

The cervical musculature was reapproximated with fine silk sutures, and the platysma and skin incisions similarly closed. The operations were performed with aseptic technique, and the animals received 300,000 units of crysticillin intramuscularly daily for 3 days.

The dogs were considered ready for the second operation and experiment several weeks after the healing of the wound. The second operation was carried out as follows:

After a 24 hour fast, two dogs prepared as already described were anesthetized with nembutal intravenously, and placed on their backs on the operating table with their necks in close juxtaposition and their muzzles in opposite directions.

A midline, longitudinal cervical incision was made in both dogs. The thyrocervical trunks and both internal jugular veins (usually of small caliber and of minor importance in the dog) were ligated and divided. The carotid arteries and external jugular veins were then isolated bilaterally in both animals and all collateral cervical branches ligated and divided, thus eliminating anastomotic circulation in the neck and insuring that essentially all blood flow through the skull occurred *via* the carotid and jugular vessels.

At this point, heparin was administered intravenously to each dog; the dosage was 4 mg. per kg. of body weight.

The external jugular vein on one side in each animal was now cut across between ligatures at about its midpoint in the neck. The cephalic end of one vein was joined to the thoracic end of the vein of the opposite dog, utilizing a polyethylene tube of the same diameter as the vein. This tube had a Blakemore cannula tied into each end to facilitate cannulation and fixation of the vein. The thoracic end of the vein of each animal was now united to the cephalic end of the vein of the other. The previously freed external jugular veins of the opposite sides in both dogs were ligated and divided. In the same way the carotid arteries in both animals were ligated, divided, and joined, the cephalic end of the artery of one animal to the thoracic end of the artery of the other, and *vice versa*. The carotid vessels on the opposite side in both animals were then divided between ligatures.

By this time the brain of each dog was receiving its blood supply almost or quite entirely from the body of its partner, whose visceral blood supply remained intact.

Venous blood specimens were drawn from the surgically exposed femoral veins in the inguinal region, a control specimen before anesthesia was begun and another after completion of the anastomoses. The Shiga toxin was then given intravenously through the same vein to one dog of the pair. Further blood specimens were withdrawn hourly from them both for the next 4 to 6 hours. Oozing from the wounds was controlled with packings of gauze. The blood loss was not measured, but in only one of the six experiments did it seem to be so great as to influence the results. This experiment, No. four, was terminated by death; the others were stopped 4 to 6 hours after the administration of the toxin. Previous experience has shown that visceral lesions regularly occur within this time interval. The experiments were brought to an end by the intravenous administration of 20 cc. of 20 per cent formalin to both dogs. If given to but one of the pair, its heart stopped at once and in 2 or 3 minutes respiration stopped in the other dog, after a few gasping movements. The one which had received the formalin continued to breathe regularly until its partner was also given it, in one case not until after 20 minutes.

Specimens of esophagus, stomach, duodenum, jejunum, ileum, and colon were fixed in formalin and stained with hematoxylin and eosin. Sections were also taken from several areas of the pancreas, adrenal glands, and kidneys and similarly treated.

RESULTS

In the unanesthetized dog, the intravenous administration of the toxin was regularly followed by the appearance of rather gross reactions consisting of fever, retching, vomiting, and diarrhea (1). Both the vomitus and the diarrheal stools were frequently bloody. In the present experiments with the dogs under anesthesia, these phenomena were not prominent. Retching was seen once and bloody diarrhea twice.

In each experiment, however, in contrast to the lack of external manifestations there was a rather characteristic sequence of events which almost exactly paralleled those previously noted in intact animals receiving the toxin intravenously. These changes occurred, not in the dog in which the toxin was given intravenously and circulated in its visceral vessels, but in its opposite partner whose brain alone received its blood supply from the circulation carrying the toxin. In these latter animals, we observed the usual hemoconcentration as measured by rise in hemoglobin and in the hematocrit reading. In addition, they showed the hyperglycemic response which, as previous studies indicated, was the result of activation of the sympathetic nervous system. Minor degrees of these changes were noted to occur in two instances in the animal which received the toxin intravenously, but the maximum alterations represented small relative changes and there was no regular pattern to these deviations from the usual course of events.

The white blood cell count showed changes which in every way corresponded to those previously noted in unanesthetized animals receiving the toxin directly. In the present instances, the leucopenia occurred in the same dogs in which the hyperglycemia and hemoconcentration occurred, namely those in which the brain alone was exposed to the toxin. During the period of leuco-

penia, there was no constant shift in the relative percentages of the polymorphonuclear leucocytes and lymphocytes; at the time at which the leucopenia disappeared and was finally replaced by a leucocytosis, the percentage of polymorphonuclear leucocytes increased at the expense of the lymphocytes. At this time there was a marked shift to the left in the blood picture, and occasional myelocytes and normoblasts appeared in the peripheral blood.

The gross anatomical lesions were most striking in the gall bladder, duodenum, jejunum, and adrenals. These lesions, in their typical and advanced degrees, were seen in the same dogs which showed the hematologic and chemical changes. The gall bladder showed edema and hemorrhage, most marked in the liver bed but present throughout the wall. The duodenum had developed mucosal congestion, edema, and superficial erosions such as we have described in detail elsewhere. The jejunal alterations were similar but less marked than those in the duodenum. The adrenal glands appeared enlarged and congested and in five of the six experiments showed hemorrhagic foci in the medulla. These changes were also noted in the partner dogs in two experiments, but only to a minor degree, amounting to some congestion in the intestinal segments and adrenal glands and some edema of the gall bladder.

The data of the experiments on the paired, cross-circulated animals are presented in the following tables:—

Experiment 1

Dog 2-70. Recipient of the toxin.
Vertebral vessels ligated: Right side, May 24, 1950. Weight, 23.0 kg.
Left side, June 28, 1950.
Weight at time of experiments, 25.0 kg.

Dog 1-31. Vertebral vessels ligated: Right side, Apr. 19, 1950. Weight, 21.0 kg.
Left side, June 7, 1950.
Weight at time of experiment, 27.6 kg.

Cross-circulation experiment done on Nov. 19, 1950.

Time	Hemoglobin		W.B.C.		P.M.N.		Lymph		Hematocrit reading		Plasma						Remarks
											Sugar		Chloride		CO_2		
	Dog 2-70	Dog 1-31	Dog 2-70	Dog 1-31	Dog 2-70	Dog 1-31	Dog 2-70	Dog 1-31	Dog 2-70	Dog 1-31	Dog 2-70	Dog 1-31	Dog 2-70	Dog 1-31	Dog 2-70	Dog 1-31	
hrs.	%	%			%	%	%	%	%	%	*mg.* %	*mg.* %	*m.eq.* /*l.*	*m.eq.* /*l.*	*vol.* %	*vol.* %	
0	122	110	12,800	12,200	64	76	36	24	50	58	85	85	90	98	38	43	Control specimen before anesthesia
0′	118	114	11,900	13,400	70	76	30	24	54	60	100	80	66	90	37	38	Control specimen on completing anastomoses. 25 cc. of Shiga toxin given dog 2-70
1	124	160	10,800	1,700	72	54	28	46	53	74	110	180	64	74	32	35	
2	130	158	10,200	2,200	68	60	32	40	56	74	85	190	68	74	24	35	
3	128	162	14,200	1,500	56	68	44	32	58	78	80	170	64	72	32	28	Dog 1-31 retching
4	132	154	16,200	8,800	74	96	26	4	54	78	70	100	70	68	32	32	
5	130	150	12,800	10,100	78	92	22	8	55	76	70	50	70	70	41	24	Dog 1-31 ceased breathing 10 min. after specimen was taken, followed in 20 min. by dog 2-70

Anatomical Findings.—Dog 2-70. Slight congestion of the duodenum, jejunum, and adrenals. Slight edema in the gall bladder bed. Clear serous fluid present in small amount in the peritoneal and both pleural cavities.

Dog 1-31. Severe congestion with petechial hemorrhages in the duodenum and proximal two-thirds of the jejunum. The gall bladder was severely edematous and hemorrhagic. Focal hemorrhages present in the adrenal medullae. Liquid and clotted blood present in the lumen of the intestine.

Experiment 2

Dog 2-67. Recipient of the toxin.
Vertebral vessels ligated: Right side, May 10, 1950. Weight, 20.0 kg.
Left side, June 14, 1950.
Weight at time of experiment, 22.0 kg.
Dog 9-0. Vertebral vessels ligated: Right side, Mar. 22, 1950. Weight, 21.5 kg.
Left side, May 31, 1950.
Weight at time of experiment, 22.0 kg.
Cross-circulation experiment done Dec. 17, 1950.

Time	Hemoglobin		W.B.C.		P.M.N.		Lymph		Hematocrit reading		Plasma						Remarks
											Sugar		Chloride		CO_2		
	Dog 2-67	Dog 9-0	Dog 2-67	Dog 9-0	Dog 2-67	Dog 9-0	Dog 2-67	Dog 9-0	Dog 2-67	Dog 9-0	Dog 2-67	Dog 9-0	Dog 2-67	Dog 9-0	Dog 2-67	Dog 9-0	
hrs.	%	%			%	%	%	%	%	%	*mg.* %	*mg.* %	*m.eq.* /*l.*	*m.eq.* /*l.*	*vol.* %	*vol.* %	
0	100	92	10,400	8,100	80	68	20	28	54	44	120	85	88	94	33	45	Control specimen before anesthesia. 4% eosinophils in dog 9-0
0′	104	100	12,200	9,600	84	68	16	32	57	50	110	125	66	72	41	42	Control specimen on completing anastomoses. 22 cc. Shiga toxin given to dog 2-67
1	114	122	9,800	4,600	64	56	26	44	58	61	115	145	74	70	33	37	Normoblasts dog 2-67, 2% dog 9-0, 36%
2	110	138	9,500	3,600	70	60	30	40	56	64	100	160	78	76	44	33	Normoblasts dog 2-67, 8% dog 9-0, 24%
3	118	134	9,880	4,900	80	72	20	28	58	62	100	165	72	72	40	29	Normoblasts dog 2-67, 6% dog 9-0, 20%
4	120	134	9,800	8,500	78	74	22	26	57	64	80	130	70	70	35	24	Normoblasts dog 2-67, 4% dog 9-0, 12%
5	118	130	14,000	16,200	90	89	10	11	57	64	85	95	74	68	34	32	Normoblasts dog 2-67, 6% dog 9-10, 12% Experiment terminated

Anatomical Findings.—Dog 2-67. Aside from slight congestion and edema of the gall bladder no visceral abnormalities were found. No ascites or pleural effusion.

Dog 9-0. Congestion, edema, and petechial lesions in the duodenum and upper jejunum. Severe confluent hemorrhages in the adrenal medullae. No pleural effusion or ascites; on the contrary, the tissues were very dry.

Experiment 3

Dog 8-29. Recipient of the toxin.
Vertebral vessels ligated: Right side, Dec. 27, 1950. Weight 25.0 kg.
Left side, Jan. 10, 1951.
Weight at time of experiment, 25.0 kg.
Dog 3-30. Vertebral vessels ligated: Right side, Dec. 20, 1950. Weight 26.0 kg.
Left side, Jan. 2, 1951.
Weight at time of experiment, 29 kg.
Cross-circulation experiment done Apr. 23, 1951.

Time	Hemoglobin		W.B.C.		P.M.N.		Lymph		Hematocrit reading		Plasma						Remarks
											Sugar		Chloride		CO_2		
	Dog 8-29	Dog 3-30	Dog 8-29	Dog 3-30	Dog 8-29	Dog 3-30	Dog 8-29	Dog 3-30	Dog 8-29	Dog 3-30	Dog 8-29	Dog 3-30	Dog 8-29	Dog 3-30	Dog 8-29	Dog 3-30	
hrs.	%	%			%	%	%	%	%	%	*mg.* %	*mg.* %	*m.eq.* /*l.*	*m.eq.* /*l*	%	%	
0	86	88	10,800	15,350	72	76	28	24	48	42	100	80	102	100	37	42	Control specimen before anesthesia
0′	96	102	9,900	13,950	80	80	20	20	49	44	110	90	96	—	34	38	Control specimen on completing anastomoses. 25 cc. Shiga toxin given to dog 8-29
1	104	122	8,600	4,500	80	80	20	20	48	55	130	180	104	102	27	38	
2	106	130	9,600	3,300	64	88	36	12	50	59	105	140	—	94	38	35	
3	104	126	9,500	8,950	88	96	12	4	50	52	90	140	100	92	26	35	
4	106	122	11,250	18,250	89	96	11	4	51	51	90	130	102	—	—	52	*
5	106	118	21,200	20,300	88	98	12	2	50	49	90	120	94	106	27	51	Experiment ended

Anatomical Findings.—Dog 8-29. Aside from slight congestion of the gall bladder no abnormalities were found.

Dog 3-30. There was moderately severe congestion and edema in the duodenum and upper jejunum with only a relatively small number of petechiae. The gall bladder was edematous and showed a moderate amount of hemorrhage in the edematous areas. The adrenals were congested and showed discrete focal hemorrhages in the medullae.

* At the time the 4th hour specimen was drawn, dog 3-30 began to rouse. It was given 4 cc. of nembutal without quieting; when 3 cc. of nembutal was given to dog 8-29 the former animal relaxed again. Both doses were given into the femoral veins.

Experiment 4

Dog 3-57. Recipient of the toxin.
Vertebral vessels ligated: Right side, Jan. 24, 1951. Weight, 21.0 kg.
Left side, Feb. 7, 1951.
Weight at time of experiment, 24.0 kg.
Dog 3-51. Vertebral vessels ligated: Right side, Jan. 17, 1951. Weight, 23.0 kg.
Left side, Jan. 31, 1951.
Weight at time of experiment, 25.0 kg.
Cross-circulation experiment done on Apr. 29, 1951.

Time	Hemoglobin		W.B.C.		P.M.N.		Lymph		Hematocrit reading		Plasma				Remarks
											Sugar		Chloride		
	Dog 3-57	Dog 3-51	Dog 3-57	Dog 3-51	Dog 3-57	Dog 3-51	Dog 3-57	Dog 3-51	Dog 3-57	Dog 3-51	Dog 3-57	Dog 3-51	Dog 3-57	Dog 3-51	
hrs.	%	%			%	%	%	%	%	%	*mg.* %	*mg.* %	*m.eq.* /*l.*	*m.eq.* /*l.*	
0	102	98	10,400	12,200	78	68	22	32	47	44	70	70	88	88	Control specimen before anesthesia
0′	108	110	8,900	10,950	82	66	18	34	48	45	80	80	100	84	Control specimen on completing anastomoses. 25 cc. Shiga toxin given to dog 3-57
1	110	130	10,050	4,800	70	56	30	44	48	56	80	105	102	104	
2	114	132	8,700	2,750	88	60	12	40	51	58	85	110	106	76	

Anatomical Findings.—Dog 3-57. Aside from a mild and diffuse congestion of all the viscera no abnormalities were noted.

Dog 3-51. The gall bladder showed congestion and moderate edema but no hemorrhage. The same was true of the small intestine and adrenals.

In this experiment there was a much larger loss of blood from the incisions than we usually observed. This was particularly true in dog 3-57. This animal became very restless shortly after the 2nd hour specimen was drawn. Dog 3-51 was given 2.0 cc. of nembutal intravenously at this time. 15 minutes later dog 3-57 ceased breathing. Dog 3-51 stopped breathing 20 minutes later.

Experiment 5

Dog 3-72. Recipient of the toxin.
Vertebral vessels ligated: Right side, Feb. 14, 1951. Weight, 23.0 kg.
Left side, Feb. 28, 1951.
Weight at time of experiment, 26.0 kg.
Dog 3-92. Vertebral vessels ligated: Right side, Feb. 21, 1951. Weight, 24.0 kg.
Left side, Mar. 7, 1951.
Weight at time of experiment, 27.0 kg.
Cross-circulation experiment done on May 6, 1951.

Time	Hemoglobin		W.B.C.		P.M.N.		Lymph		Eosinophil		Hematocrit reading		Plasma				Remarks
													Sugar		Chloride		
	Dog 3-72	Dog 3-92	Dog 3-72	Dog 3-92	Dog 3-72	Dog 3-92	Dog 3-72	Dog 3-92	Dog 3-72	Dog 3-92	Dog 3-72	Dog 3-92	Dog 3-72	Dog 3-92	Dog 3-72	Dog 3-92	
hrs.	%	%			%	%	%	%	%	%	%	%	*mg.* %	*mg.* %	*m.eq.* /*l.*	*m.eq.* /*l.*	
0	106	110	9,300	13,200	66	76	30	22	4	2	37	40	85	85	76	90	Control specimen before anesthesia
0′	108	110	9,200	12,400	76	76	22	20	2	4	38	42	90	150	104	92	Control specimen on completing anastomoses. 30 cc. Shiga toxin given to dog 3-72
1	114	122	8,500	7,100	60	80	34	14	6	6	40	47	125	160	100	102	
2	112	128	9,100	3,900	68	75	32	25	—	—	40	50	85	130	98	108	
3	118	128	11,500	6,900	88	88	10	12	2	—	42	52	65	130	90	98	
4	110	132	20,300	20,500	94	92	5	8	1	—	38	55	85	90	102	92	
5	116	128	23,100	26,150	94	96	6	4	—	—	42	52	70	75	80	98	Experiment terminated

Anatomical Findings.—Dog 3-72. The gall bladder showed slight edema but no hemorrhagic changes. There was slight congestion of the small intestine and adrenals.

Dog 3-92. The gall bladder was markedly edematous and hemorrhagic. The duodenum and upper jejunum were congested, edematous, and showed numerous petechiae. The adrenals were congested and showed hemorrhagic medullae.

Experiment 6

Dog 2-34. Recipient of the toxin.
Vertebral vessels ligated: Right side, Mar. 14, 1951. Weight, 24.0 kg.
Left side, Mar. 28, 1951.
Weight at time of experiment, 28.0 kg.
Dog 2-65. Vertebral vessels ligated: Right side, Mar. 21, 1951. Weight, 25.0 kg.
Left side, Apr. 4, 1951.
Weight at time of experiment, 27.0 kg.
Cross-circulation experiment done on May 30, 1951.

Time	Hemoglobin		W.B.C.		P.M.N.		Lymph		Eosinophil		Hematocrit reading		Plasma				Remarks
													Sugar		Chloride		
	Dog 2-34	Dog 2-65	Dog 2-34	Dog 2-65	Dog 2-34	Dog 2-65	Dog 2-34	Dog 2-65	Dog 2-34	Dog 2-65	Dog 2-34	Dog 2-65	Dog 2-34	Dog 2-65	Dog 2-34	Dog 2-65	
hrs.	%	%			%	%	%	%	%	%	%	%	*mg.* %	*mg.* %	*m.eq.* /*l.*	*m.eq.* /*l.*	
0	90	90	19,400	12,100	84	80	16	17	—	3	42	40	90	85	104	96	Control specimen before anesthesia
0′	98	104	19,800	13,300	78	74	22	22	—	8	45	45	100	135	72	98	Control specimen on completing anastomoses. 30 cc. Shiga toxin given to dog 2-34
1	98	110	18,700	5,900	84	66	12	28	4	6	45	48	115	200	88	102	
2	108	124	17,100	7,350	90	74	8	20	2	6	46	52	90	240	102	98	
3	100	120	21,500	12,400	84	88	14	6	2	6	44	50	90	150	98	96	
4	96	116	22,200	12,700	84	88	12	12	4	2	44	50	85	160	102	96	Experiment terminated

Anatomical Findings.—Dog 2-34. Congestion noted in the small intestine and gall bladder. No other changes noted.
Dog 2-65. The gall bladder and duodenum were edematous, congested, and hemorrhagic. There were confluent hemorrhages in the adrenals. The thymus appeared enlarged and showed focal hemorrhages. The adrenal lesions were located in the medullae.

DISCUSSION

Before any attempt can be made to evaluate the significance of the data the validity of the method used must be established (9). It is obvious that if there were only a partial separation of the cerebral from the visceral circulation in either of the paired dogs it would be impossible to draw any conclusions from these experiments. It would seem worth while to differentiate anatomical from physiological separation. We do not believe that the former was realized in our experiments. To have attempted to achieve that goal would have involved procedures which were sufficiently drastic in themselves to have produced the lesions. Our data, however, present sufficient internal evidence to indicate that physiological separation was realized within the limits required by the pathogenetic mechanisms apparently involved.

It will be noted that in our experiments the animals recovered completely between operations. This was shown not only by their behavior but by their gain in weight. This eliminated the possibility that the operative procedures produced the anatomical lesions through the shock involved in the manipulations. This complication was doubtless avoided through the development of a collateral circulation within the spinal canal and skull, by way of anastomoses

existing between the vertebral branches of the intercostal and lumbar arteries and veins, and the corresponding branches of the basilar vessels. In the interval between ligating the second pair of vertebral vessels and the final experiment, there may also have occurred increasing opening of the collaterals between the thyrocervical trunk and its branches and the branches of the carotid vessels. These, however, were eliminated in the final experiment when the trunk was ligated and cut, as were also the branches of the common carotid arteries and jugular veins below the level of the bifurcation into internal and external carotids.

While the anatomical separation of the two circulations was not absolutely complete, it is obviously possible that the pressure relationships within the brain circulation may have been such as to prevent any noteworthy admixture of blood between the brain of a dog and its own visceral blood supply under the conditions of the final experiment. The pressure in the carotid arteries and in the circle of Willis is much higher than that present in the minute vertebral branches of the intercostal and lumbar arteries. The latter were so small, that, even at a time when the collateral circulation had presumably developed, they were extremely difficult to find, even though specifically searched for. Under such conditions admixture of visceral blood, while anatomically possible, would not occur.

In a real sense, under the conditions of our experiments one animal serves as a control for the other. Thus, the reactions of the dog receiving the toxin *via* its femoral vein and delivering it to the brain of its partner serve as a control in evaluating the reactions of said partner. If a physiologically significant amount of visceral blood mixed with the cerebral in the dog which received the toxin intravenously, it would be expected that that animal would develop to some degree the hematologic, biochemical, and anatomical changes seen in its partner. This did not occur in our series of experiments, and we therefore feel justified in concluding that the experimental separation of the cerebral from the visceral circulation was adequate for our test.

From the facts as stated it follows that Shiga toxin produces its systemic and local anatomical effects through some primary action in the brain. More precise localization is not possible by the present method. From the sequence of events observed during the time of development of the lesions, the logical assumption would follow that, if the toxin acted to stimulate some area of the brain, the area involved would include the hypothalamus and probably the adjacent tuber cinereum and pituitary gland. On the other hand, if the primary action of the toxin were one of depression, the site of action would necessarily involve those higher centers which control the hypothalamic nuclei in the sense of Jackson (10). In this relation the concept of the blood brain barrier offers a possible solution to the problem. While the original observations and theories of Lewandowsky (11) and Goldmann (12), as modified by

the later work of Friedemann (9), Broman (13), and others, have not as yet arrived at a complete understanding of the mechanisms involved in brain permeability, certain observational data at least have been consistently reported. With but few exceptions, those vessels directly and intimately concerned with the nutrition of the brain parenchyma possess a characteristic permeability which differs from those of other organs. The exceptions mentioned include at least one which may concern the present problem. The permeability of the parenchymal capillaries supplying the pituitary gland, tuber cinereum, and adjacent hypothalamus is such that dyes which are retained within the parenchymal circulation elsewhere in the brain diffuse through these apparently more permeable vessels into the surrounding brain tissue (12, 13). These findings lend support to the hypothesis that the toxin may also escape into these areas and thus initiate the diffuse sympathomimetic discharge whose peripheral manifestations we have been studying.

The leucopenia, which appeared during the sympathomimetic response elicited by the toxin, was not an intrinsic component of this response since it persisted when the hemoconcentration, hyperglycemia, and anatomic changes were prevented by ergotamine tartrate or tetraethyl ammonium chloride. The fact that it was observed not only in intact animals but also in the dogs of the cross-circulation experiments which developed lesions, would indicate that it also was due to some reaction in the central nervous system elicited by the toxin. The data of White (14) and his coworkers show that this leucopenia can be explained by the increased secretion of adrenotropic hormone by the anterior pituitary gland and its action on the adrenal cortex. In our experiments in which the visceral lesions were prevented (3, 4) the leucopenia occurred in the absence of any manifestations of sympathomimetic activity. Similarly, in the present experiments the leucopenia occurred in the dogs whose central nervous systems were supplied with blood from partners who showed no evidence of sympathomimetic activity. Furthermore, the visceral circulation of the dogs manifesting sympathomimetic overactivity flowed through the brains of their mates and the latter failed to show a leucopenia. These observations would indicate that the leucopenia is also the result of an action of the toxin mediated through the central nervous system and that the mechanisms involved are independent of those concerned in the production of the visceral lesions.

SUMMARY

An analysis of the mechanisms involved in the pathogenesis of the visceral lesions produced by Shiga toxin in the dog has indicated that the structural changes are produced by a vascular mechanism which is under control of the sympathetic nervous system. Paralysis of the latter at the myoneural junction or in the ganglia by means of drugs served to prevent the tissue changes as

well as the hyperglycemia and hemoconcentration characteristic of the sympathomimetic response. The observations indicated that the toxin acted *via* a mechanism located in the central nervous system.

Further studies of the action of Shiga toxin by means of cerebral cross-circulation are reported here. In these the cerebral blood flow of one dog derived from the circulation of the trunk and limbs of another. Injection of the toxin into the femoral vein of this latter dog resulted in the appearance of visceral lesions, hemoconcentration, and hyperglycemia in the former whose brain only received blood containing the toxin. The resulting visceral changes were identical with those observed in the intact unanesthetized dog which had received the toxin directly. The observations indicate that, in dogs, Shiga toxin produces its characteristic visceral lesions *via* the central nervous system. Possible mechanisms are discussed.

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