IMMUNOLOGICAL AND PATHOLOGICAL PHENOMENA RELATED TO SUBSTANCES FROM TISSUES OF *ASCARIS LUMBRICOIDES*[*]

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NUMEROUS investigators have studied the reactions observed in experimental animals following inoculations of extracts from various animal parasites. The reports deal chiefly with the intravenous injection of extracts or suspensions of whole parasites and a description of resulting symptoms, but with little or no emphasis on the mechanism of the ensuing toxic reactions.

In the helminthiases, excretions and secretions from living parasites and substances from parasitic forms which disintegrate in the tissues are being constantly absorbed into the blood of the host. The similarities or differences between toxic reactions produced by metabolic products and by toxic substances from dead worms, is a topic which also has been superficially studied.

Studies performed by inoculating extracts from the tissues of *Ascaris lumbricoides var suum,* i.e., cuticle, muscle, intestine, eggs, sperm, and coelomic fluid, into experimental animals, have revealed that all of these materials contain a substance which produces fundamentally the same reaction in each animal species, and that the mechanism of the toxic reaction is apparently the same. The results also suggest that the toxic substance within the tissues of the parasite are also present in its metabolic products.

MATERIALS AND METHODS

Preparation of extracts from Ascaris *tissues and tests for serum agglutinins.* Adult *Ascaris lumbricoides* var. *suum* were obtained in fresh condition and the tissues were dissected as described in a previous report

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(Oliver-González, 1943). Each tissue was washed in distilled water and stored in the freezer at −20°C to −25°C, until ready for use; the material for extraction was suspended in 0.04 molar tris-hydroxymethyl amino-ethane buffer, and homogenized in a 100 ml. all-glass electric-run tissue grinder. The homogenate was centrifuged at 3,000 r.p.m. for 15 min. and the centrifugate was discarded. Sodium chloride was then added to make a 0.5 per cent solution, followed by the addition of 5 to 7 volumes of 95 per cent alcohol. A precipitate was recovered after storage overnight at 6°C, which was dried by various washings in alcohol and ether. The dry material was weighed and suspended in 0.85 per cent salt solution for injection.

The effect of the tissue extracts on the α_2 isoagglutinins in human serum of Groups B and O was determined by adding known amounts of the substance to small volumes of the serum. The serum-extract mixture, in 10 × 60 mm tubes was incubated at 37°C for one hour and tested with human cells of Group A_2 in order to detect the α_2 agglutinin titer. Untreated serums, diluted with saline to the same volume of the treated serums, were used as controls. The titers were observed under the stereoscopic microscope at a magnification of ×20.

Animals injected with the ascaris tissue extracts were tested for agglutinins against their own erythrocytes. One tenth ml. volume of the undiluted and of serial dilutions of the plasma were incubated with 0.05 ml. of a one per cent suspension of the erythrocytes. The plasma-erythrocyte suspension was incubated for 1 hour at 37°C and then read under the stereoscopic microscope as above.

Animal Inoculations

All inoculations of dogs were done intravenously, using the veins of the front legs. The required amount of extract was suspended in 10 ml. of saline and homogenized in an all-glass grinder.

Dogs were weighed and blood samples were obtained before inoculation and immediately after death. Complete blood and platelet counts, observations on the coagulation time, and serological tests for agglutinins were performed on all blood samples.

The animals were carefully observed after injection for reactions, and autopsies were done on as many dogs receiving each tissue extract as possible. Portions of heart, lungs, spleen, liver, kidneys and mesenteric lymph nodes were fixed in Zenker-formol fixative for histopathological studies.

Guinea pigs of both sexes weighing from 450 to 650 gm. were bled from the heart and the serum was tested for α_2 agglutinins. Using a 1 ml.

syringe and 22-gauge needle, the inoculations were made intracardially, after suspending the required amount in 1 ml. of saline. One group of normal animals was inoculated with each of the tissue extracts. Another group of guinea pigs was fed with 10,000 to 15,000 infective ascaris eggs. Ten to 15 days after feeding, the animals were injected intracardially with the ascaris tissue extracts. The guinea pigs in both groups were observed carefully for reactions, and were killed 24 hours after inoculation. Tissue studies were made as with the dogs.

The mice utilized in this study were of an A strain weighing from 22 to 30 gm. The tail vein was used for inoculations of the tissue extracts suspended in 1 ml. of saline. One group of normal mice was inoculated with each of the ascaris tissue extracts. Another group was immunized with human erythrocytes of Group A_2. Each animal received 1 ml. of a 5 per cent suspension of cells in saline, intraperitoneally, for 3 consecutive days in a week, and after a rest period of 5 days, the injections of A_2 erythrocytes were continued for another 3 days. Ten days after the last immunizing dose, mice were injected intravenously with the required dose of tissue extract. The mice in both groups were observed for reactions, and were killed 24 hours after injection of the tissue extract. Blood was collected, and the serum tested for α_2 isoagglutinins.

Normal mice injected with A_2 erythrocytes only and with tissue extract only were also killed as controls. Tissue studies were performed as with the dogs and guinea pigs.

Results

Toxic reactions and pathological changes observed in dogs inoculated with extracts from ascaris tissues. All dogs inoculated intravenously with the ascaris tissue extracts had α_2 agglutinins in the serums. The titers varied from 1:32 to 1:128. Immediately after inoculation, the animals exhibited symptoms similar to anaphylaxis. These were chiefly vomiting, urination, defecation and difficult breathing, followed by collapse and death. Animals usually died within the first hour after inoculation. The minimum lethal doses required to cause death are presented in Table I. Animals which received a dose less than that became resistant to further inoculations. It is evident from Table I that all of the tissues contain the substance which provoked the anaphylactoid symptoms and death.

The most notable postmortem findings were intense congestion of the liver and intestinal blood vessels as seen from both the mucosal and serosal aspects. Microscopically, in some organs the erythrocytes within blood vessels were agglutinated, with loss of their usual shape and sharpness of outline. This was particularly extensive in the liver, which showed

intense congestion. The spleen showed moderate congestion and depletion of lymphocytes in the follicles. Pathological studies on these and other tissues will be considered in greater detail in another report.

Hematological studies revealed a marked drop in platelets and white blood cells. The erythrocytes increased, probably as a result of hemoconcentration due to the loss of liquids. The blood lost its coagulability indefinitely.

The plasma of inoculated dogs agglutinated the animal's own erythrocytes. Agglutination was observed at low titers, after incubation for 1 hour

TABLE I

MINIMUM AMOUNT OF EXTRACT FROM TISSUES OF *Ascaris lumbricoides var suum* CAUSING DEATH OF DOGS WITH α_2 AGGLUTININS IN THE SERUM

Number of Dogs	Tissue Extract Inoculated	Average Mgm/Kilogram	Reactions	
			Anaphylactoid and Death	Main Pathological Findings
8	Cuticle	0.3	Positive	Liver Congestion
6	Cuticle	0.5	Positive	Liver Congestion
7	Cuticle	1.0	Positive	Liver Congestion
3	Egg	0.5	Positive	Liver Congestion
4	Egg	1.0	Positive	Liver Congestion
2	Intestine	18.0	Positive	Liver Congestion
2	Intestine	15.0	Positive	Liver Congestion

(Table II) at 37°C, but tended to disappear after prolonged incubation for longer periods of time.

Observations on Guinea Pigs and Mice. Normal guinea pigs exhibited no symptoms after inoculation with each of the tissue extracts. Such manifestations as scratching of the nose, coughing or gasping for breath, which are readily observable in sensitized guinea pigs, did not appear in these animals. However, the histopathological studies revealed that the guinea pigs in both groups of animals which had a high titer of α_2 isoagglutinins in the serum, had developed as marked a congestion of the liver as was observed in the dogs (Table III).

No anaphylactoid reactions appeared in 19 guinea pigs infected with ascaris 10 to 15 days previously, and intracardially injected with 5 to 15 mg/kg of extract of ascaris cuticle. These animals at autopsy had numerous ascaris larvae in the lungs.

No anaphylactoid reactions were observed in any of the mice of the various groups, i.e., those immunized against A_2 erythrocytes, those injected intravenously with the tissue extracts, and the ones injected with

TABLE II

AUTOAGGLUTININS AGAINST DOG'S OWN ERYTHROCYTES AFTER INTRAVENOUS INOCULATION WITH EXTRACTS FROM *Ascaris* TISSUES CONTAINING THE α_2 INHIBITING SUBSTANCE

Dog Number	Tissue Extract Inoculated	Before Inoculation							After Inoculation, at the Time of Death						
		Undiluted Plasma	Dilutions						Undiluted Plasma	Dilutions					
			2	4	8	16	32	64		2	4	8	16	32	64
524	Cuticle*	Negative							3+	2+	+	+	0	0	0
525	Cuticle	Negative							4+	2+	+	+	+	+	0
535	Cuticle	Negative							2+	+	+	0	0	0	0

* Results of tests in plasma of dogs inoculated with extracts of egg, intestine, and sperm were the same as those from dogs inoculated with cuticular extract.

the tissue extracts after immunization with A_2 erythrocytes (Table IV). These animals behaved as the non-sensitized controls.

Marked differences were observed, however, in the tissues from the animals immunized against human Group A_2 erythrocytes, and then challenged with the tissue extracts, as compared with the liver sections from the animals injected with each of the other above substances. No congestion or agglutinated corpuscles were observed in the mice immunized against A_2 erythrocytes or those injected intravenously with ascaris tissue extracts, but intense congestion of the liver, with large coagulated

TABLE III

INTRACARDIAC INOCULATION OF THE EXTRACT FROM *Ascaris* CUTICLE INTO NORMAL GUINEA PIGS*

Number of Guinea Pigs	Titer Against A_2 Erythrocytes Before Inoculation	Mgm/Kilogram	Reactions	
			Anaphylactoid and Death	Histological Liver
6	Negative	10	No	Normal
6	Negative	15	No	Normal
5	Negative	20	No	Normal
4	1:4	20	No	Normal
6	1:8	20	No	Congestion
6	1:32	20	No	Congestion

* Similar results were obtained with the extracts from the other ascaris tissue extracts.

masses of erythrocytes, was observed in those mice in which agglutinins had been developed in the serum and which were then injected with ascaris extracts.

DISCUSSION

The fatal reactions produced in dogs after inoculation of each of the ascaris tissue extracts do not differ in any way from the reactions previously described by Rocha e Silva and Graña (1946). The substance used by these investigators was an extract from the whole adult worm described as mainly nitrogenous, and as a proteose of high molecular weight. The symptoms and tissue changes observed in both studies were indistinguishable from that observed in anaphylactic shock. Clinically, there is vomiting, urination, defecation and collapse. Pathologically, engorgement of the liver, decreased blood coagulation, diminished platelets, and leukopenia were observed constantly. In our study, the substance which causes the above reaction is present in varying amounts in all ascaris tissues and is easily extracted with a buffer at pH 8.9. The buffer used in this study (0.04 molar-tris-hydroxymethyl aminoethane) does not affect

TABLE IV

INTRAVENOUS INOCULATION OF THE *Ascaris* CUTICLE EXTRACT INTO NORMAL MICE AND MICE PREVIOUSLY IMMUNIZED AGAINST HUMAN GROUP A_2 ERYTHROCYTES

Normal					Inoculated with A_2 Erythrocytes				
Number of Mice	Titer Against A_2 Erythrocytes	Mgm/ Kilogram	Reactions		Number of Mice	Titer Against A_2 Erythrocytes	Mgm/ Kilogram	Reactions	
			Anaphylactoid and Death	Histological Liver				Anaphylactoid and Death	Histological Liver
5	Negative	25	No	Normal	3	1:32 to 1:64	25	No	Congestion
5	Negative	50	No	Normal	5	1:16 to 1:64	50	No	Congestion
5	Negative	50	No	Normal	5	1:16 to 1:64	50	No	Congestion

tissue enzymes, and will extract water soluble substances.

The substance isolated from the ascaris tissue is by no means a pure entity. Investigations are under way to isolate the active principle and thus perform chemical studies.

The substance which causes fatal reactions in dogs is shown to be related to the A_2 isoagglutinogen in human Group A_2 erythrocytes, by the fact that when added to human serums of Groups O and B, it inhibits the α_2 isoagglutinins in the serum. Each of the ascaris tissue extracts has been found to posses such property (Table V). The secretions and excre-

TABLE V

MINIMUM AMOUNTS OF EXTRACTS FROM *Ascaris* TISSUES REQUIRED TO INHIBIT THE α_2 ANTIBODY IN HUMAN SERUM OF GROUPS B AND O

Tissue from Which Substance Was Extracted	Milligrams of Substance per cc of Serum	α_2 Titers Before and After Adding Substance			
		Group B Serum		Group O Serum	
		Before	After	Before	After
Cuticle	1.0	1:32	0	1:64	0
Egg	3.0	1:32	0	1:32	0
Coelomic Fluid	1.3	1:64	0	1:32	0
Intestine	1.0	1:16	0	1:64	0
Sperm	2.1	1:64	0	1:32	0

tions of animal parasites and bacteria also inhibit the α_2 isoagglutinins in human serums (Oliver-González, 1954). This suggests that the A_2 isoagglutinogen-like substance is of metabolic importance to the infecting parasites, since it is found in all tissues of the worms, and it is present in the metabolic products.

There is some evidence suggesting that one of the factors responsible for the death of the dogs is intravascular agglutination of erythrocytes. In the first place, plasma of animals inoculated with the tissue extracts agglutinated the animal's own erythrocytes. This did not occur before inoculation. Secondly, the sections of liver and other organs showed agglutination of erythrocytes in large clumps. Rocha e Silva and Porto (1946) reported agglutinated platelets and leukocytes in their animals that died after the intravenous injection of ascaris extracts, but they make no mention of agglutinated erythrocytes. Agglutination of erythrocytes, as well as of platelets and leukocytes, is therefore an important finding in the possible mechanism of death.

From the above studies it is not definitely established what the antigen and the antibody are that react to cause agglutination. Apparently the

α_2 isoagglutinins normally present in the animal sera constitute the antibody and the A_2 isoagglutinogen-like substance present in the ascaris tissue extract constitutes the antigen. It has to be postulated, therefore, that the A_2 substance from the worms adheres to the erythrocytes and that the agglutinin reacts with the red cells which have the homologous agglutinogen on their surfaces. The above studies merely suggest that such may be the case, but lack evidence to show that the substance introduced goes totally or in part to coat the erythrocytes. Finer techniques of experimentation are necessary, particularly tagging of the substance with radioisotopes which would facilitate following the fate of this substance in the animal's body.

Other indirect evidence suggests that the α_2 isoagglutinins play a major role in the agglutination of erythrocytes. Intravenous inoculation of normal mice with the α_2 agglutinin-inhibiting substance present in the ascaris tissue extracts cause no congestion of the liver. However, the liver of mice immunized with human A_2 erythrocytes, and therefore with the α_2 agglutinins in the serum, showed marked congestion of the liver after injection of the tissue extracts. Therefore, as in the dogs, the α_2 agglutinins in the serum and the A_2 substance introduced through inoculation were requisites for the reaction.

The infection of guinea pigs with ascaris, which is manifested by the presence of larvae in the lungs, apparently did not sensitize the animals to the tissue extracts. Although the metabolic products of these larvae may constitute a source of antigen to stimulate the formation of α_2 antibodies, these antibodies could not be detected in the serum. This may have been due to the fact that the antigenic stimulus was inadequate. Although numerous larvae were present in the lungs, their stay in this organ is short, since they migrate into the intestine and are rapidly expelled. The reactions observed in the dogs, guinea pigs and mice are, therefore, not due to previous sensitization with ascaris larvae, but to some other mechanism such as explained above.

As has been shown in previous studies, the A_2 isoagglutinogen-like factor of infectious agents does not inhibit the α_1 or β agglutinins in human serums, as tested *in vitro* (Oliver-Gonzalez, 1954). Mice should be immunized with human erythrocytes of Groups A_1, B and O, and then tested by injection with the ascaris tissue extracts. The *in vivo* reaction may not be specific to the A_2 erythrocytes.

The tissues from ascaris contain other antigenic substances which upon injection into rabbits lead to the development of antibodies related to other types of immunological reactions (Oliver-Gonzalez, 1943), but in the studies here presented we are dealing with a factor which is common

to all tissues, but which is responsible for one type of reaction. (There are, however, other substances in the tissues responsible for other types of antigen-antibody reactions.)

Summary

1. Extracts were prepared from cuticle, muscle, intestine, egg, sperm, and coelomic fluid dissected from adult *Ascaris lumbricoides* var. *suum*. Each material was extracted in 0.04 molar tris-hydroxymethyl-aminoethane, and the soluble substance was then precipitated with 95 per cent alcohol.

2. Each extract, when injected intravenously into dogs, caused an anaphylactoid type of reaction followed by rapid death. The main pathological findings were congestion of the liver and intestinal veins. There was a marked decrease in leukocytes and platelets, and incoagulability of the blood.

3. Histopathological studies showed agglutination of erythrocytes, observed particularly in sections of the liver. The blood plasma of the inoculated dogs agglutinated the animal's own erythrocytes.

4. Guinea pigs inoculated intracardially with the ascaris tissue extracts did not exhibit anaphylactoid symptoms, but marked congestion of the liver was seen in the animals with a high α_2 isoagglutinin titer in the serum. No congestion of the liver was observed in the guinea pigs without α_2 antibodies.

5. Guinea pigs infected with ascaris by feeding embryonated eggs prior to inoculation of the tissue extracts did not respond with the anaphylactoid reaction. This suggests that such a reaction is not related to previous sensitization with migrating ascaris larvae.

6. It is suggested that, among other mechanisms, autoagglutination of the erythrocytes may have an important role in the reactions of animals inoculated with the ascaris tissue extracts; also, that autoagglutination is mediated by α_2 isoagglutinins, normally present in the animals sera, which react with the injected antigen. It is also postulated that the antigen, when injected, adheres to the erythrocytes.

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