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Serological Relationships between Collagenase and the A_2-Isoagglutinogen-like Substance of Animal Parasites.* (26450)

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Recent studies have indicated that the A_2-isoagglutinogen-like substance (A_2IS) in animal parasites may be associated with a collagenase complex. Thus the sera from rabbits artificially immunized against the A_2IS agglutinate human erythrocytes of Group O treated with tannic acid and exposed to collagenase prepared from various sources(1). Also, collagenase, like A_2IS inhibits the a_2 isoagglutinins in human sera of Groups B and O. The A_2IS utilized in these studies was extracted from the cuticle of *Ascaris* and was used as a crude material. A purified A_2IS has been prepared which shows more activity than the crude material with respect to inhibition of a_2 isoagglutinins and which is also serologically related to collagenase.

Lewert *et al.*(2) have shown that collagenase-like enzymes are present in the larval forms of *Schistosoma mansoni, Ancylostoma caninum* and *Strongyloides ratti.* Collagen-like substances have also been described from various animal parasites(3,4) particularly from the cuticle of *Ascaris,* which constitutes a good source of A_2IS. Collagenase, therefore, is apparently present in animal parasites either as a component of their tissues or in their metabolic products.

Materials and methods. Animal inoculations. Male rabbits weighing from 1.5 to 2 kilos were artificially immunized with various substances as described previously(1). Dogs weighing 4.5 to 5 kilos were each injected intravenously with 2 to 3 mg of A_2IS, collagenase, pepsin and trypsin† per kilo of body weight. The animals were observed closely for reactions to the various substances and were bled before injection and immediately after death. Dogs were autopsied and tissues fixed for histopathological studies immediately after death.

Preparation of purified A_2IS. The crude and dry material obtained from *Ascaris* cuticle, according to the procedure described elsewhere(1) was reextracted with triple distilled water at 4°C, centrifuged for 30 minutes at 9000 × G (International refrigerated centrifuge PR-1) and the supernatant dialyzed exhaustively against triple distilled water in a cold room for a period of 2 days with several changes. The dialyzed solution was then lyophilized. The lyophilized material will be referred to as "purified A_2IS".

Total protein analyses were carried out by the method of Lowry *et al.*(5); total carbohydrate and cold trichloracetic acid-precipi-

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† Collagenase A—Nutritional Biochemical Corp., Cleveland, Ohio.

Collagenase B—Mann Research Laboratories, New York.

Pepsin and Trypsin—Difco Laboratories, Inc., Detroit, Mich.

table-protein-bound-carbohydrates were determined colorimetrically according to Siebert(6). Electrophoresis was performed with "OXOID" Cellulose acetate strips, as described by Kohn(7).

Agglutination tests. The substances to be tested for inhibition of the a_2 isoagglutinins in human sera of Groups B and O were first dissolved in the specific pH buffered saline for each substance, then diluted 2-fold in 0.1 cc volumes of 0.85% sodium chloride. One-tenth cc volume of Group O or B human sera was then added to the 0.1 cc volume of the respective dilution of substance, and incubated at 37°C for 30 minutes. Each serum-substance mixture was then diluted 2-fold, after which 0.1 cc of a 2% suspension of washed human erythrocytes of sub-group A_2 was added. The serum erythrocyte mixture was left at room temperature for 1 hour and examined for agglutinated erythrocytes under the stereoscope at × 20. Substances tested for a_2 inhibitory activity were crude and purified A_2IS, collagenase, pepsin and trypsin.

Sera from dogs injected with various substances were tested for agglutinins against their own erythrocytes by mixing 0.1 cc volumes with 0.5 cc of a 2% suspension of the dogs' own erythrocytes before and after injection which had been washed 3 times in saline. The mixtures in tubes were incubated at 37°C for 60 minutes, then portions were examined under the microscope at × 340.

Hemagglutination tests were performed on human and animal sera following Boyden's technic(8), with slight modifications, as used by Kagan(9) in his studies concerning serological diagnosis of helminthic infections. Ten mg of the enzymes collagenase, trypsin and pepsin were dissolved in 10 cc of buffered saline at pH 8.8 and 5.5 respectively. The Group O human erythrocytes treated with tannic acid were exposed for 10 to 15 minutes to the above concentration of substances or to further dilutions, if necessary. Reference positive sera were obtained from rabbits immunized against A_2IS. As a daily procedure, known positive and negative sera were tested against Group O tannic acid treated erythrocytes and exposed to the antigens. If the tests were positive to high titers with the known positive, and no agglutination was observed with the known negative sera, all of the remaining unknown sera were then tested. Sera from rabbits inoculated with A_2IS were tested against O-collagenase, O-trypsin and O-pepsin, and with the homologous O-A_2IS erythrocytes as well.

The sera of rabbits immunized against A_2IS were treated with powdered collagenase, trypsin, pepsin and crude A_2IS, and were then tested for agglutinins against O-A_2IS erythrocytes. Five milligrams of the substance was added to 0.5 cc of the serum mixed well by means of a 1 cc tissue homogenizer, then incubated at 37°C for one hour. After incubation the mixture was centrifuged for 5 minutes at 1200 × g. Treated and untreated sera were then tested for agglutinins against O-A_2IS erythrocytes.

Agar gel double diffusion tests were performed using the rapid slide technic described by Yakulis and Heller(10). Sera from rabbits immunized against collagenase were tested against a 1:1,000 solution of the purified A_2IS and of collagenase. Normal rabbit sera were also tested against collagenase as controls. The slides were incubated at 37°C for several days and examined macroscopically for presence of bands of precipitate.

Results. Biochemical characteristics of the purified A_2IS. Preliminary determinations have revealed that only 22% of the crude material from the cuticle was water-soluble under the experimental conditions described. The electrophoretic patterns of purified A_2IS indicated the presence of one slow-moving homogeneous protein (Fig. 1a) accompanied by a negligible impurity. Under the same electrophoretic conditions, the pattern of commercial collagenase showed mainly 2 fractions (Fig. 1b): the slow-moving one corresponding to the A_2IS protein. Therefore the electrophoretic similarities at pH 8.6 of these 2 proteins are apparent.

The gross chemical composition of the A_2IS protein is shown in Table I. The high content of carbohydrate in A_2IS suggests that it is a glycoprotein.

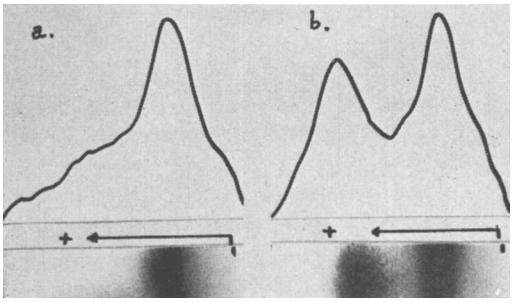

FIG. 1, a and b. Electrophoretic patterns of purified A_2IS and of collagenase showing similar slow-moving protein fractions.

Inhibition of a_2 isoagglutinins by various substances. Of all substances tested, collagenase and the purified A_2IS were the ones which inhibited the a_2 isoagglutinins in human sera of Groups B and O, when using the lowest concentration of material. Activity of the collagenase preparations varied from 4 to 20 μg per cc of serum. That of purified A_2IS was similar to collagenase, 4 μg per ml of serum. Pepsin and crude A_2IS inhibited at a level of 350 and 2500 μg, respectively, while trypsin had no effect (Table II).

TABLE I. Chemical Composition of Purified A_2IS.

Total protein	Total carbohydrate	Protein-bound carbohydrate	Free carbohydrate
	% W/W*		
17	83	66.3	22.7

* Expressed as % of dry wt.

Hemagglutination reactions with rabbit antisera. Sera from rabbits immunized against A_2IS reacted to high titers with the homologous O-A_2IS erythrocytes and with O-collagenase, but not with O-pepsin or O-trypsin

TABLE II. Inhibition of a_2 Isoagglutinins in Human Serums of Groups B and O by Various Substances.

Enzyme or substance used	a_2 isoagglutinin titer: Before treatment	After treatment μg/cc of serum	
Crude A_2IS	1:128	2,500	0
Purified A_2IS	1:128	4	0
Collagenase A	1:128	4	0
" B	1:128	20	0
Pepsin	1:128	350	0
Trypsin	1:128	3,000	1:128
"	1:128	5,000	1:128

treated erythrocytes. Treatment of these sera with A_2IS and with collagenase absorbed the antibody completely. Treatment of the sera with trypsin resulted in partial absorption, but there was no absorption with pepsin (Table III).

TABLE III. Absorption of Hemagglutinins by Various Substances.

Substance	Hemagglutinin titer against O-A_2IS erythrocytes: Before treatment of serum	After treatment of serum
Collagenase A	1:20,480	0
Crude A_2IS	1:40,960	0
Purified A_2IS	1:40,960	0
Pepsin	1:10,240	1:10,240
Trypsin	1:40,960	1: 1,280

Agar gel precipitin reactions. The rabbit anticollagenase sera reacted with a 1:1,000 suspension of collagenase in saline as shown by the presence of one thin and one coarse band of precipitate. These antisera also reacted with the 1:1,000 solution of purified A_2IS from *Ascaris* cuticle. One major band could be clearly seen which coalesces with the coarse band formed with collagenase, (Fig. 2). Normal rabbit sera did not react with collagenase or with the purified A_2IS.

Reactions to intravenous injection. The dogs which received injections of crude A_2IS and of collagenase died within 4 hours thereafter. The animals manifested symptoms of an anaphylactoid reaction, such as urination, defecation, vomiting and death. Urine and feces of the animals injected with collagenase were of a dark red color, indicating presence of blood. Sera from these dogs had agglutinins against the animals' own erythrocytes. Agglutinins in animals injected with collagenase were higher than those injected with A_2IS (1:32 dilution of serum as compared with 1:2). Dogs injected with trypsin and pepsin did not manifest anaphylactoid reactions, and survived.

Histopathological studies of liver and spleen of dogs injected with A_2IS and collagenase also revealed marked congestion and agglutinated erythrocytes. Observations on changes in the kidneys and other organs will be reported later.

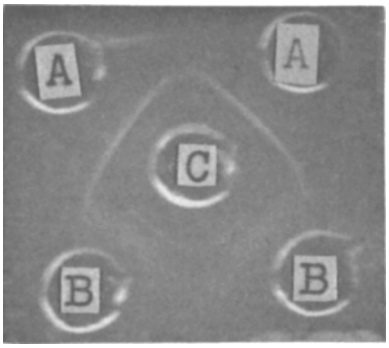

FIG. 2. Agar gel diffusion slide showing precipitin bands formed when anticollagenase sera (C) reacted against A_2IS (A) and collagenase (B).

Discussion. These results indicate that the A_2IS from animal parasites is serologically related to collagenase. Rabbit antisera against A_2IS reacted positively with O cells coated with collagenase. No such reactions were observed with these sera against O cells coated with pepsin or trypsin. Furthermore, treatment of the rabbit antisera with collagenase absorbed the hemagglutinins for O-A_2IS erythrocytes.

Further evidence for the serological relationship between the A_2IS substance and collagenase is seen in the agar gel double diffusion tests. The purified A_2IS formed 2 bands of precipitate when tested against the anticollagenase sera. The most prominent band coalesces with the coarse band observed when the same sera are tested against the homologous antigen collagenase.

Another relationship is indicated by the electrophoretic patterns of both substances, which shows a similar slow moving component.

Close serological relationships between A_2IS and collagenase are thus indicated by: 1. Specific action and degree of inhibitory activity against a_2 isoagglutinins in human sera of Groups O and B. 2. Cross reactions as observed in hemagglutination tests using A_2IS rabbit antisera against Group O-collagenase erythrocytes. Also the antibody against each substance was absorbed by the heterologous as well as by the homologous antigen. 3. Cross precipitin reactions observed in agar diffusion tests when anticollagenase rabbit sera are tested against the A_2IS. 4. Death of dogs when both substances are injected intravenously. The animals die from an anaphylactoid reaction with similar reactions and histopathological observations.

Collagenase, as prepared commercially from species of *Clostridia* is not a pure substance and may have other active impurities. This is shown by the fact that 2 bands of precipitate are observed when tested against homologous antisera. One of the bands is coarse, which is the one coalescing with the major band formed when the serum is tested against the purified A_2IS. This indicates that the A_2IS may be present as part of the collagenase complex or as a contamination. The serological relationships discussed above indicate, however, that A_2IS may be an important part of the collagenase complex. Further studies on the antigenic and enzymatic properties of collagenase and A_2IS will undoubtedly clarify this aspect.

The presence of related antigenic substances such as the A_2IS found in *Ascaris* and in *Clostridia* is an illustration of a common antigen between helminths and bacteria which may be important in serological diagnosis and host's reactions.

Death occurs when dogs are injected intravenously with collagenase or A_2IS. The mechanism of death is not understood, although autoagglutination of erythrocytes may be a factor. Apparently the substance adsorbs *in vivo* onto the dogs' red cells and thus a reaction occurs between the a_2 agglutinins in the dog plasma and the dogs' erythrocytes. The erythrocytes apparently change in antigenicity as a result of adsorption of the new antigen. This is indicated by the fact that the serum agglutinates erythrocytes obtained after inoculation of the A_2IS and collagenase and not those obtained before inoculation. *In vivo* adsorption of collagenase or like substances onto erythrocytes may take place during infection with organisms which secrete this substance. This may be the mechanism for autoagglutination of erythrocytes observed during some infectious processes.

The anaphylactoid reaction observed in dogs after intravenous inoculation of collagenase and of A_2IS suggests that these sub-

stances are concerned in hypersensitivity reactions, in which autoagglutination of erythrocytes and other pathological phenomena caused by allergens are involved.

Summary. The A_2 isoagglutinogen-like substance (A_2IS) from animal parasites has been prepared in a purified form from crude extracts of the cuticle of *Ascaris lumbricoides* var suum. Preliminary chemical studies indicated that this substance is a glycoprotein. The A_2IS is related to collagenase or a collagenase complex, as shown by cross hemagglutination reactions between anticollagenase sera prepared in rabbits, and the A_2IS substances adsorbed onto tannic acid treated Group O human erythrocytes. Also A_2IS reacts with anticollagenase sera in agar diffusion slides with formation of one band of precipitate. The two substances are also immunologically related as shown by the fact that they inhibit the a_2 isoagglutinins in human serum of Groups O and B when comparable amount of each substance is used per ml of serum. A_2IS and collagenase also cause anaphylactoid symptoms and death when injected intravenously into dogs. It is suggested that collagenase or substances in the collagenase complex are present in infectious organisms which may be associated with hypersensitivity.

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Renal Na-Reabsorption and O_2-Uptake in Dogs During Hypoxia and Hydrochlorothiazide Infusion. (26451)

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It seems well established that sodium reabsorption in the kidney is an active process, which requires the bulk of the oxygen consumed by the kidney. In recent reports of experiments on the dog kidney, it has been shown that there is a fixed relation between sodium reabsorption and oxygen consumption (mEq Na/net mmol O_2). Thaysen, *et al.*,(1) using different dogs with large individual variations in rate of sodium reabsorption, found a fairly constant Na/net O_2 ratio of 28.5. Following acute hemorrhage, so that no glomerular filtration and, therefore, no sodium reabsorption occurred, basal renal oxygen consumption was determined to be 1.0 μmol/g kidney/min. Deetjen and Kramer(2) found a ratio of 24 over a wide range of sodium reabsorption in the same dog. By compressing the aorta above the renal artery, they were able to decrease the tubular sodium load by decreasing the glomerular filtration rate with a resulting drop in sodium reabsorption. By extrapolating from these data, basal renal oxygen consumption, *i.e.*, when no sodium is reabsorbed, was calculated to be 1.5 μmol/g kidney/min.

The present study was undertaken with the following aims: (1) to decrease active reabsorption of sodium and oxygen consumption in the kidney by means of low arterial oxygen pressure or administration of hydro-

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