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Antigenicity of Hyaluronic Acid.* (23197)

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Studies of antigenicity of hyaluronic acid indicate that it is not antigenic by itself(1) and when coupled chemically with protein it does not act as a hapten(2). Recent work of Glynn and Holborow(3) reports the production of complete antigens from vegetable polysaccharides (chondroitin sulfate) to autoantigens by group A hemolytic streptococci (4). Although Glynn and Holborow have since indicated that they have been unable to repeat their work and that observed antibodies were directed against blood group like substances and not against chondroitin sulfate itself(5), the question of whether hyaluronic acid, and other tissue polysaccharides might become autoantigenic is important in possible interpretations of the pathogenesis of rheumatic fever. It is the purpose of this paper to report attempts to render hyaluronic acid antigenic.

Materials. Hyaluronic acid was prepared from human umbilical cords according to method described by Jeanloz and Forchielli (6), and digested with pepsin but not with trypsin.‡ Trypsin digestion was eliminated to avoid the necessity of lowering the pH with possible denaturation of hyaluronic acid. Remaining proteins were eliminated by Sevag's procedure(7) which was performed at least 10 times. Sulfur-containing compounds were removed by treating the hyaluronic acid solution twice with ammonium sulfate and pyridine(6). Streptococcal hyaluronic acid was prepared from the N.Y. 5 strain of group A hemolytic streptococcus, employing the method of Pierce and White(8). The small amount of residual protein was removed by Sevag's procedure. A crude extract of umbilical cords was prepared according to the method of McClean *et al.*(9). Bacteria used as antigens were 3 strains of group A beta hemolytic streptococci belonging to serological types 1, 4 and 14, and a strain of hemolytic *Staphylococcus aureus*. In preparation of materials for inoculation, the bacteria were incubated for 18 hours at 37°C in 100 ml of brain heart infusion broth, centrifuged and taken up in 25 ml of 0.85% saline. Half of this bacterial suspension was employed as antigen without exposure to hyaluronic acid, and half was centrifuged and resuspended in 10 ml of a solution of cord hyaluronic acid, 1 mg hyaluronic acid/ml of 0.85% saline. Bacteria were not exposed to streptococcal hyaluronic acid. Half of the bacteria were incubated at 37°C for 1 hour with hyaluronic acid *before* being heat-killed and half *after* being heat-killed. Then these suspensions were washed 4 times in 0.85% saline. A portion of the suspension in contact with hyaluronic acid before killing was not washed to test any possible effect of washing on antigenicity. Careful controls were observed to insure pure cultures. The cord hyaluronic acid and streptococcal hyaluronic acid injected without bacteria contained 1 mg and 1.3 mg hyaluronic acid respectively/ml of

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‡ Had trypsin digestion been done it is possible that the numerous attempts to remove proteins by Sevag's procedure might have been unnecessary. It seems likely that each Sevag procedure removed with it some of the hyaluronate, thus accounting for the very small yield of 255 mg from a starting material of 2000 g of human umbilical cords which had been stored in acetone.

solution. Group A, type 1 strain had been recovered from a patient with pharyngitis about 3 weeks preceding onset of rheumatic fever; this strain produced streptolysin "O", streptokinase and hyaluronic acid, but no hyaluronidase. Strain A-14 was recovered from a patient with pharyngitis about 2 weeks preceding onset of rheumatic fever and produced streptolysin "O", streptokinase, hyaluronic acid and a small amount of hyaluronidase after 7 days' incubation. The source of group A, type 4 was unknown but it produced relatively large amounts of hyaluronidase. The strain of *Staphylococcus aureus* was recovered from a patient with infected skin ulcer.

Methods. Intravenous inoculation procedures. Young adult rabbits weighing between 5½ and 6½ lb were injected intravenously in the marginal ear vein with antigens indicated in Table I. Three rabbits were used to test each antigen. Each rabbit received 1 injection of 0.5 ml of antigen daily for 3 days during the first week. They were allowed to rest 4 days, then received 1 ml daily for 3 days, followed by 4 days' rest. This routine was followed for 4 more weeks. The dose of hyaluronic acid antigens was adjusted so that each injection after the first week contained 1 mg of hyaluronic acid. After 6 weeks the rabbits were reinjected following the same schedule for 3 more weeks. The seventh course of injections was given subcutaneously in a dose of 0.5 ml; all subsequent injections consisted of 1 ml intravenously. Each rabbit received a total of 27 injections. The rabbits were bled from the ear vein 1 week after completion of the third course of injections, 2 days after completion of the sixth course and 8 days after the ninth course. The serum was separated and Merthiolate added to a strength of 1:10,000 before storing at 4°C-8°C. *Intramuscular and subcutaneous inoculation procedures.* Antigens given by these routes are listed in Table I. The hyaluronic acid or crude extract content of each antigen was 1 mg/ml. The dose was 0.5 ml 3 times weekly for an average of 23 injections. Procedure for administering these antigens differed from that described above in that half the animals received bacteria which were heat-killed before contact with hyaluronic acid or crude extract and half received bacteria which were killed after contact. In this way the possible effect of any hyaluronidase elaborated by group A, type 4 hemolytic streptococci could be observed. Each rabbit was bled before any antigen was given, at 2 week intervals during antigen administration, and 10 days following last injection. *Test for precipitating antibodies.* An optimal proportion technic was employed in which sera were tested undiluted, 1:4, 1:8 and so on to a dilution of 1:64. The antigens, 1 mg hyaluronic acid or crude extract/ml of 0.85% saline, were diluted identically. Each serum dilution was tested with each antigen dilution beginning with undiluted serum plus all antigen dilutions, etc. Dilutions of sera and antigens were made in small serological tubes. The serum was first taken up in a capillary tube to a height of about 1 cm, then overlaid by an equal volume of antigen. The tubes were incubated at 37°C for 2 hours after which they were placed in refrigerator at 4°C-8°C overnight. They were observed for evidence of precipitate during and after 37°C incubation and after 4°C-8°C incubation. *Test for antigroup-specific C carbohydrate.* Streptococcal C carbohydrate was extracted according to the procedure of Swift, Wilson and Lancefield(10). Serum from each rabbit which had received streptococci intravenously was tested for presence of antibodies against the group-specific C carbohydrate. *Test for antitype-specific M protein.* Sera from rabbits which had received streptococci by various routes were tested for precipitating antibodies against M protein. M protein was extracted from each strain of hemolytic streptococcus according to method described by Lancefield(11). *Test for skin sensitivity.* Each rabbit was skin-tested with 0.1 mg in 0.1 ml of the antigen which it received: umbilical cord hyaluronic acid, streptococcal hyaluronic acid, or crude extract. Injection sites were observed immediately and at 24, 48 and 72 hours for erythema and induration. *Test for non-precipitating, skin-sensitizing antibodies.* A test for non-precipitating, skin-sensitizing antibodies was performed with sera of rabbits which had received either

TABLE I. Antigens Injected, Route of Administration, and Antibody Response.

Materials injected	No. of animals inj.	Route of inj.	Antibody determinations: Pptn. ab. against hy. ac. cord	Pptn. ab. against hy. ac. strep.	Pptn. ab. against cr. ext.	Skin sensitivity	Skin-sens. non-pptn. ab.	Anti C CHO ab.	Anti M prot. ab.
Streptococcus A-14	3	I.V.	0			0	0	+	+
" A-1	3	"	0			1-e.	0	+	+
Staphylococcus aureus	3	"	0			"	0		
Hy. ac. (umbilical cord)	3	"	0			2-e.	0		
" " (streptococcal)	3	"		0		1-in. 1-e. & in.	0		
Streptococcus A-14 + hy. ac.	3	"	0			0	0	+	+
" A-1 + " "	3	"	0			1-e.	0	+	+
Staphylococcus aureus + hy. ac.	3	"	0			"	0		
Streptococcus A-14 + hy. ac. unwashed	3	"	0			0	0	+	+
Hy. ac. (umbilical cord)	1	I.M.	0			0	0		
" " + A-4, killed before contact	1	I.M.	0					+	+
	1	S.C.	0					+	+
" " + " contact before killed	1	I.M.	0			0		+	+
	1	S.C.				0		+	0
" " + staph., killed before contact	1	I.M.	0			0			
" " + " contact before killed	1	S.C.	0			0			
Crude extract	1	I.M.			0	0			
" " + A-4, killed before contact	1	"			0	1-e. & in.		+	+
" " + " contact before killed	1	"			0	"		+	+
" " + staph., killed before contact	1				0	0			
Total No. of rabbits	39								

Hy. ac. = Hyaluronic acid; Cr. ext. = Crude extract from human umbilical cords.

Killed before contact = Bacteria were heat-killed before exposure to substrate. Contact before killed = Bacteria were exposed to substrate, then heat-killed. Numbers in column under skin sensitivity refer to No. of rabbits showing erythema, induration or both.

I.V. = Intravenous; I.M. = Intramuscular; S.C. = Subcutaneous; in. = induration; e. = erythema.

streptococcal or umbilical cord hyaluronic acid intravenously with or without bacteria, employing the Prausnitz-Küstner technic. Test sites were prepared by injecting 0.1 ml of each rabbit's serum intradermally into a normal rabbit. Forty-eight hours later, each prepared site was injected in its center with 0.01 ml of the hyaluronic acid which had been employed to vaccinate the animal supplying the serum. The results were read in 30, 45 and 60 minutes.

Results. None of the rabbits had any noticeable physical reactions following injections of antigens. Immunologic results are shown in Table I. None of the rabbits developed the slightest trace of precipitating antibodies against either umbilical cord hyaluronic acid or streptococcal hyaluronic acid. The sera of rabbits which had received crude extract showed a heavy precipitate which appeared very rapidly after overlaying with the crude extract solution. However, in testing sera from these rabbits before they had received any crude extract, the same heavy precipitate was observed. Therefore, whatever this precipitate was, it was not due to precipitating antibodies acquired during administration of crude extract.

Skin tests in rabbits inoculated intravenously, using hyaluronic acid from umbilical cords or from streptococci as the skin test material, revealed slight erythema lasting for 2 days about the injection site in 1 rabbit which had received streptococcus A-1, and in 1 which had received *Staphylococcus aureus.* A large area of erythema, fading gradually in 3 days, was observed about the injection site in 1 rabbit which had received umbilical cord hyaluronic acid, and a small area of erythema lasting 1 day was seen in another rabbit which had received the same antigen. A small raised area of induration, 2-3 mm in diameter, lasting for 2 days, and a similar "lesion" plus a small amount of erythema was observed in 2 rabbits which received streptococcal hyaluronic acid. Erythema fading in 3 days was observed in 1 rabbit which received streptococcus A-1 plus hyaluronic acid, and in 1 which received *Staphylococcus aureus* plus hyaluronic acid. Erythema 10 x 10 and 10 x 12 mm was observed at 1 hour in 2 rabbits which received crude extract plus streptococcus A-4. In 24 hours the erythema measured 10 x 11 and 7 x 7 mm and there were small areas of induration 4 x 4 and 3 x 3 mm. The induration remained for several days. The significance of all the skin reactions is not clear. Had induration appeared at all injection sites there would have been no question that these reactions were indicative of a reaction of the tuberculin type, but its absence in all but 4 animals leaves some doubt as to the significance of erythema alone as a measure of sensitivity.

The results of the tests for non-precipitating, skin-sensitizing antibodies were entirely negative during the 1 hour period of observation.

Sera from rabbits which had received streptococci incubated with hyaluronic acid were tested for precipitating antibodies against M protein of types 1, 4 and 14. After the last injection, large amounts of precipitating antibody against type-specific M protein were present in sera of all but 1 of the rabbits which had received streptococcus A-1 or A-14 alone or in combination with hyaluronic acid. A very small amount of precipitin against type 4 M protein was present in sera of 4 of 6 rabbits which received type 4 streptococci with hyaluronic acid or crude extract. Precipitating antibodies against an acid extract of type 4 containing group-specific C carbohydrate were present in moderate amounts in sera of all rabbits which received type 4. The ability of this strain of group A, type 4 to stimulate production of anti-M antibodies in rabbits must be of a low order.

Discussion. The results of these experiments indicate that by itself, hyaluronic acid derived from human umbilical cord and from group A hemolytic streptococci does not stimulate in rabbits the production of precipitating or non-precipitating, skin-sensitizing antibodies, nor does umbilical cord hyaluronic acid become a hapten as does agar(3) when exposed to group A hemolytic streptococci or *Staphylococcus aureus.* The same can be said for crude extract of umbilical cord. The results were the same for all 3 routes of administration. Why hyaluronic acid did not stimulate production of these

antibodies is not explained by these experiments, but it is of interest that Glynn and Holborow§ also failed to produce antibodies to hyaluronic acid using these technics. Possibly the hyaluronic acid was depolymerized or was not bound tightly to bacterial cells and was washed off in preparing the antigens, although these same workers made the observation that live group A streptococci, having once been in contact with agar or alginate solution, acquired antigenicity for these haptens, which was retained despite repeated washing of the live cocci(4). Furthermore, in the present experiment 3 rabbits which received immunizing doses of streptococci and hyaluronic acid which were not washed, did not develop antibodies against hyaluronic acid.

The production of antitype-specific M protein and antigroup-specific C carbohydrate antibodies in rabbits inoculated with streptococci is evidence that these rabbits had received a dose of at least 2 antigens large enough to stimulate antibody production. If hyaluronic acid were antigenic, it would seem that there had been ample time for antibodies to appear. The possibility that the hyaluronic acid may have been destroyed by hyaluronidase produced by the streptococci does not seem valid because type 1 did not produce this enzyme and type 14 did so only in very small amounts after a 7 day incubation period. Rabbits which received hyaluronic acid and crude extract which had been incubated with hyaluronidase-producing type 4 streptococci both before and after being heat-killed did not produce antibodies either, so apparently hyaluronidase did not interfere with any potential antigenicity of the connective tissue components.

The question of production of skin sensitivity is not so clear. The presence of small areas of induration and erythema in the skin of 2 rabbits receiving streptococcal hyaluronic acid would suggest that these rabbits might have developed some tuberculin-like sensitivity. The same might be said of the 2 rabbits which received crude extract plus type 4 streptococci and developed erythema and slight induration 24 hours after test injection.

Summary. Antigens were prepared from human umbilical cord hyaluronic acid, streptococcal hyaluronic acid, group A hemolytic streptococci and *Staphylococcus aureus* and these same bacteria incubated with human umbilical cord hyaluronic acid. Rabbits were injected intravenously, intramuscularly and subcutaneously with these antigens. Neither precipitating nor non-precipitating, skin-sensitizing antibodies against hyaluronic acid developed. Anti C carbohydrate and anti M protein antibodies were produced in the animals which received streptococci, indicating that the rabbits had received enough of at least 2 antigens, one a carbohydrate the other a protein, to stimulate antibody production. The development of skin sensitivity to hyaluronic acid was suggested but by no means proved. These experiments furnish further evidence that hyaluronic acid is probably not antigenic by itself, nor does it act as a hapten capable of adsorption to group A hemolytic streptococci or *Staphylococcus aureus.*

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