INFECTIOUS MYXOMATOSIS OF RABBITS

Studies of a Soluble Antigen Associated with the Disease

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The occurrence of a soluble, serologically active substance in materials from rabbits with infectious myxomatosis has been reported (1); both serum and extracts of infected skin, obtained from animals acutely ill with the disease and freed from virus by filtration, contain a precipitinogen which reacts specifically with serum of animals recovered from fibroma and myxoma. Study on this soluble substance or substances has been continued in the hope of learning its significance in myxomatosis, *viz.*, what part it plays in the production of immunity to infection and in the excitation of neutralizing, precipitating and agglutinating antibodies. Such an investigation would obviously be simplified if the precipitinogen were free from serologically inert material. The purpose of the present paper is to record the effect of certain physical, chemical, and biological agents on the precipitinogen and to indicate the direction in which purification may be pursued. In addition, observations on the immunological and serological phenomena associated with the partially purified antigen are presented.

Materials and Methods

Filtered Extract of Infected Skin.—Rabbits inoculated intradermally in numerous sites over the backs and flanks with a bacteriologically sterile emulsion of infectious skin are sacrificed, generally on the 6th day, when the resulting lesions are fully developed but not yet necrotic. The skin is macerated in a meat grinder, covered with physiological salt solution, and stored at 0°C. Ether is added to prevent bacterial growth. After periods, varying from several days to several months, the fluid is cleared of large particles and passed through a Seitz filter. Filtration is always slow. The filtrate is a reddish brown, highly viscous solution, rich in the mucoid material typically present in lesions produced by the virus of myxoma.

Dermal Filtrate.—Another kind of filtrate is obtained, as previously described by Rivers and Ward (1), in the preparation of suspensions of purified elementary bodies of myxoma from dermal pulp of rabbits infected by means of scarification. The infected skin is scraped while covered with dilute disodium phosphate-citric acid buffer solution, pH 7.2, and the resultant suspension, after preliminary horizontal centrifugation, is spun in an angle centrifuge. The supernatant fluid, removed from the sedimented elementary bodies, is passed through a Seitz filter. The filtrate is a clear, slightly yellow fluid.

Filtrate of Serum.—A third source of soluble antigen is the serum of rabbits acutely ill with myxoma as a result of extensive infection of the skin. Serum is separated from blood, obtained by cardiac puncture on the 5th or 6th day after inoculation, and filtered through a Seitz pad.

Immune Sera.—Sera collected from rabbits which had been repeatedly inoculated with myxoma virus after recovery from an infection with fibroma virus (1) have been pooled and used in a dilution of 1:8 for precipitin reactions. Convalescent serum from a rabbit which survived an attack of myxomatosis that resulted from infection by contact was also used; this serum was supplied by Dr. R. F. Parker. The specificity of serological reactions has been controlled by use of sera from normal rabbits or from rabbits recovered from a vaccinal infection.

Precipitin and Agglutinin Reactions.—Precipitin and agglutinin reactions have been conducted as previously described (1): graded dilutions of soluble antigenic material to be tested were mixed with constant amounts of diluted immune or control sera; for the agglutination tests graded dilutions of serum were added to a properly diluted suspension of elementary bodies. All mixtures were incubated in closed racks overnight at 50°C.

EXPERIMENTAL

Filtrates prepared in the manner described are free from virus but contain soluble material or materials which precipitate in the presence of immune sera. Different preparations vary in their content of soluble antigen and in general a lower titer is obtained with dermal filtrate than with filtered extract of skin or with filtered serum. Consequently, most of the work has been conducted on the serologically active material derived from the last two sources. Experience with all three types of filtrate, however, both freshly prepared and after storage, indicates that there is a common or closely related antigen in each.

Effect of Physical, Chemical, and Biological Agents on the Soluble Material

The occurrence of soluble antigen or antigens, separate from infectious agents, has been noted in connection with bacteriophagy (2),

yellow fever (3), vaccinia (4), psittacosis (5), influenza (6), and myxomatosis (1). A soluble antigen that is either completely or almost completely resistant to inactivation at a temperature of 50–56°C. has been described in all the virus diseases mentioned except myxoma. A second soluble antigen, which is inactivated at a temperature of 50°C. is present in filtrates made from tissues infected with vaccine virus. In view of these facts, an investigation of the stability of the soluble antigen or antigens of myxoma was indicated and the effect of heat was examined first.

Small amounts of a filtrate were placed in separate tubes, raised to the temperature desired, allowed to remain at that temperature in a water bath for different periods of time, and then cooled rapidly. Frequently, considerable opalescence appeared in the contents of tubes incubated at relatively high temperatures. When this occurred, the precipitate was removed before tests were made for the presence or absence of serologically active material in the supernatant fluids. In Table I the results of typical experiments with all three types of active filtrate are summarized.

It is apparent from Table I that the precipitinogen is usually inactivated by a temperature of 56°C. for 1 hour; at times it may be inactivated in 15 minutes. No heat-stable soluble antigen separable from the virus has been found in myxoma and in that respect it differs from other virus diseases studied. This lability renders impossible the purification of the active principle through coagulation of serologically inert protein by means of heat.

The range of hydrogen ion concentrations in which the precipitinogen retains its activity was next investigated.

The nature of filtrates derived from macerated skin and from serum is such that the hydrogen ion concentration cannot be changed readily by the use of buffer solutions. Adjustment of the pH is accomplished, therefore, by addition of normal or tenth-normal NaOH and HCl. The accuracy of colorimetric methods which have been used as routine in the estimation of pH values was controlled on several occasions by determinations with a glass electrode.

Samples of 5 cc. of filtered serum were placed in separate tubes and adjusted to pH 3.0, 4.0, 4.5, 5.0, 7.0, and 9.0, respectively, by addition of appropriate amounts of normal HCl or NaOH solutions. The tubes were stored at +5°C. for 2 hours, and then spun in an angle centrifuge for 1 hour at 0°C. to remove the slight precipitates which had formed in the tubes at pH 4.0, 4.5, and 5.0. The supernatant fluids were readjusted to pH 7.0 and brought to equal volumes (8.0 cc.) with physiological saline solution. Results are summarized in Table II.

The results recorded in Table II show that activity of the precipitinogen in filtered serum was lost at pH 3.0, and slightly decreased

TABLE I

Effect of Heat on Serological Activity of Soluble Antigen of Myxoma

Type of filtrate	Temp.	Time exposed	Dilution of antigen					
			1:2	1:4	1:8	1:16	1:32	1:64
	°C.							
Extract of skin	37	1 hr.	++++	++++	+++	±	−	
	56	15 min.	++++	++++	±	−	−	
	56	1 hr.	±	−	−	−	−	
	Untreated		++++	+++	++	±	±	
Filtrate of serum	37	1 hr.	±	±	++++	++++	++++	±
	56	15 min.	±	±	−	−	−	−
	56	30 min.	−	−	−	−	−	−
	Untreated		±	+	++++	++++	++++	±
Dermal filtrate	56	1 hr.	−	−	−	−	−	
	Untreated		+++	+++	+++	++	−	

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.

TABLE II

Serological Activity of Filtered Serum, Obtained from Acutely Ill Myxomatous Rabbits, after 4 Hours at Various Hydrogen Ion Concentrations

pH	Dilution of antigen				
	1:4	1:8	1:16	1:32	1:64
3.0	±	−	−	−	−
4.0	++++	++++	++	±	−
4.5	++++	++++	++++	−	−
5.0	++++	++++	++++	++	−
7.0	+	++++	++++	++++	±
9.0	++++	++++	++++	+++	−

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.

at pH 4.0 and 4.5, but that between pH 5.0 and 9.0 little change occurred. Similar results have been obtained with filtered extracts of

infected skin in which the antigen is only slightly affected by remaining overnight at pH 4.0 at 0°C.; however, a dark brown inactive precipitate formed which could be removed as shown in experiments on purification.

A precipitate which forms between pH 4.0 and 5.0, and which is greatest in amount at about pH 4.5, appears to be different from the one just mentioned. This material is readily soluble when the pH is changed either to below 4.0 or above 5.0, but if it is removed and washed with saline buffered at pH 4.5 only a small part redissolves at pH 7.0. The experiment detailed below and recorded in Table III indicates that at pH 4.5 some active material is precipitated but that the supernatant fluid also retains activity.

One of two 5 cc. samples of filtered extract of infected skin was adjusted to pH 4.0 and the other to pH 4.5 by addition of small amounts of half-normal HCl. A precipitate appeared immediately in each sample but was coarser and heavier at pH 4.5. The precipitates were removed after the mixtures had remained for 2 hours at +5°C. and each was washed 3 times with physiological saline solution buffered at pH 4.0 and 4.5 respectively. The washed sediments were resuspended in physiological saline solution, pH 7.0; only a small part of each sediment went into solution and the residues, which were insoluble, were discarded. The original supernatant fluids containing the components of the filtrate of skin which were soluble at pH 4.0 and 4.5 were restored to pH 7.0 by means of small amounts of half-normal NaOH. Since the final volumes of the four samples were unequal, Table III indicates only approximate titers.

These investigations and others on the behavior of the precipitinogen in acid and alkaline solutions suggest that the isoelectric point of the active material is near pH 4.5. However, experiments reported so far were conducted only in solutions containing normal concentrations of salt; hence, the effect of change in concentration of electrolytes became of interest. A preliminary experiment showed that the specific substance precipitates completely in a half saturated solution of ammonium sulfate. Smaller concentrations of ammonium sulfate have not been effective in completely precipitating the active principle when filtrates are treated at pH 7.0. However, the experiment detailed below shows that 30 per cent saturation of ammonium sulfate at pH 4.5 does completely precipitate it.

100 cc. of filtered extract of infected skin (titer 1:32) which had been partially purified by removal of material insoluble at pH 4.0 and by removal of the albumin

fraction by means of ammonium sulfate were divided into two equal portions; one was adjusted to pH 4.5, the other was kept at pH 7.0. Sufficient saturated ammonium sulfate solution was added to bring the concentration in each portion of filtrate to 30 per cent. A precipitate appeared in both samples immediately, but, after standing at +5°C. overnight, the sample at pH 4.5 was observed to have a heavy precipitate in a yellowish supernatant fluid while that at pH 7.0 had a moderate precipitate in a reddish brown supernatant fluid. Both sediments were removed by centrifugation and dissolved in dilute buffer solution. The redissolved sediments and both supernatant fluids were brought to pH 7.0 and dialyzed overnight against running water. The final volume of each of the supernatant fluids after dialysis was 115 cc. The material precipitated by 30

TABLE III

Serological Activity of Soluble and Insoluble Fractions of Filtered Extract of Infected Skin Obtained at pH 4.0 and 4.5

pH	Fraction	Dilution of antigen				
		Undiluted	1:2	1:4	1:8	1:16
4.0	Soluble	++++	++++	++++	++	±
	Insoluble	−	−	−	−	−
4.5	Soluble	++++	++++	++++	−	−
	Insoluble	++++	+	−	−	−
7.0	Untreated	+++	++++	++++	++++	±

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.
See text for preparation and use of fractions in the reactions.

per cent saturation of ammonium sulfate at pH 4.5 was contained in 24.5 cc., while that precipitated at pH 7.0 was in 11 cc.

Results of titration of the four fluids obtained in the above experiment are given in Table IV. Most of the active principle remains soluble in a concentration of 30 per cent saturated ammonium sulfate at pH 7.0, but at pH 4.5 practically all of it is precipitated. This is additional evidence that the isoelectric point of the active principle is near pH 4.5.

It has been observed that the heat-stable antigen of vaccinia (S) remains soluble in 80 per cent alcohol when the reaction of the solution is slightly acid, but is insoluble when the alcoholic solution is neutral (7). A similar behavior on the part of the soluble antigen of

myxoma would facilitate its separation from serologically inactive proteins which are present in all crude preparations and especially in extracts of skin. However, the antigen is completely precipitated in the cold, without inactivation, from serum and from extracts of

TABLE IV

Serological Activity of Soluble and Insoluble Fractions of Extract of Infected Skin Obtained by Treatment with 30 Per Cent Ammonium Sulfate at pH 4.5 and 7.0

pH	Fraction	Dilution of antigen					
		1:2	1:4	1:8	1:16	1:32	1:64
7.0	Insoluble	−	++++	++++	++	−	−
	Soluble	++	++++	++++	++	−	−
4.5	Insoluble	−	±	++++	++++	++++	+
	Soluble	−	−	±	−	−	−

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.
See text for preparation and use of fractions in the reactions.

TABLE V

Serological Activity of Filtered Serum, Obtained from Acutely Ill Myxomatous Rabbits, after Contact with Formaldehyde (U.S.P.) for 18 Hours

Formaldehyde	Dilution of antigen				
	1:2	1:4	1:8	1:16	1:32
per cent					
None	+++	++++	++++	±	−
0.5	+	++++	++++	±	−
1.0	−	++	+	−	−
2.0	−	−	−	−	−

Fibromyxoma serum used throughout in dilution 1:8.
+'s indicate degree of precipitation.
Formaldehyde was removed by dialysis before tests were performed.

infected skin by the addition of 9 volumes of absolute alcohol either at pH 6.0 or at 7.2. Under these conditions the serologically inactive proteins as well as the active principle are precipitated. Acetone also precipitates the active fraction, but no appreciable separation from inert material is effected.

Treatment of the soluble substance with formaldehyde in concentration greater than 0.5 per cent leads to loss of activity (Table V).

The effect on the antigen of a 5 per cent solution of cysteine hydrochloride, which had been brought to pH 7.0, was investigated; this mild reducing agent does not inactivate filtrates after storage overnight at +5°C., nor does its presence prevent inactivation of the antigen by heat.

The effect of a mixture of proteolytic enzymes, namely, commercial trypsin, on the activity of filtered serum and extract of infected skin was investigated. Diminution in the specific serological activity of the filtrates has not followed their treatment with 1 per cent active commercial trypsin which had been previously extracted with petrol ether and ethyl ether while in the dry state.

Partial Purification of Soluble Antigen of Myxoma

With the information recorded above at hand, namely, that the antigen can be partially separated from associated serologically inert material by methods of differential precipitation, based on its solubility at different pH values and in different concentrations of ammonium sulfate, we attempted to purify the soluble antigen in filtrates obtained from serum of infected animals and from extracts of infected skin. An experiment on the purification of the antigen from each type of filtrate will be presented in detail, after which the characteristics of the resulting materials will be described.

85 cc. of filtered serum, which had a precipitin titer of 1:64, were mixed with an equal volume of saturated ammonium sulfate. The precipitate which formed was removed, dissolved in dilute buffer solution, and dialyzed against running water until tests were negative for sulfate ions. The small amount of flocculent precipitate which was present in the dialysate was washed in distilled water; only a part of this was soluble in physiological saline, and, because it was found to be only slightly active, the solution was discarded. Sufficient sodium chloride to make a physiological concentration was dissolved in the fraction containing the soluble dialysate. Then this fraction was adjusted to pH 4.5, after which sufficient ammonium sulfate was added to make a final concentration of 30 per cent. The precipitate which formed was removed after storage for 18 hours at +5°C., dissolved in dilute buffer solution at pH 7.0, and dialyzed overnight against running water. Again a slight precipitate appeared in the dialyzed solution; it was also discarded after it had been shown to possess only slight

serological activity. A precipitin titration carried out with a small amount of the 56 cc. of solution obtained after dialysis gave approximately the same end-point as had the original filtrate of serum from infected animals. The solution was adjusted to pH 8.0 and 1 cc. of a 1 per cent solution of commercial trypsin which had been extracted with petrol ether and ethyl ether was added. After incubation at 37°C. for 1 hour and at +5°C. overnight, the solution was again precipitated at pH 4.5 in a concentration of ammonium sulfate equal to 30 per cent saturation in order to remove as much trypsin as possible. The resultant fine white precipitate was dissolved in dilute buffer solution, pH 7.2, and dialyzed until sulfate ions were no longer detectable; then it was desiccated from the frozen state. 0.5741 gm. of white powder were obtained.

The dry antigen, purified in the manner described, dissolved completely in 6 cc. of distilled water forming an opalescent solution; then sufficient sodium chloride was added to make a physiological solution. The precipitin titer of this solution, calculated on the basis of the above dry weight of partially purified material, was approximately 1:25,000. When a 1:100 solution of this material was examined spectroscopically,[1] absorption bands indicating the presence of tryptophane, tyrosine, and phenylalanine were found; there was no spectroscopic evidence of the presence of nucleic acid. After the solution had been stored at +5°C. for a week, a small amount of a very fine white precipitate appeared and was removed by ultracentrifugation at 20,000 R.P.M. for 20 minutes; the precipitin titer of the supernatant fluid remained the same. The solution was further stored in a frozen state for 3 weeks and then desiccated again. A dry residue weighing 0.4582 gm. was obtained. After still further storage in the dry state at 0°C. for several weeks, a portion of the preparation was removed for study. Two 5 mg. samples were negative for yeast and thymonucleic acid, respectively; a 10 mg. sample gave a delayed, faint but definitely positive Molisch reaction; the dried material contained 15.4 per cent nitrogen.[2] The precipitin titer on an aliquot of the redissolved material was of the order of 1:10,000 (dry weight). Another portion of the 0.4582 gm. of dry residue was removed after additional storage, in a sealed tube, for 2 months at room temperature. The material now failed to react with fibromyxoma serum even when a solution of it contained 1 part dry weight in 750. Moreover, this inert substance no longer showed the solubilities of the original antigen for it now precipitated readily when the pH of the solution was in the neighborhood of 5.4, but remained soluble at pH 4.5.

It is apparent from the record of purification of soluble antigen contained in serum that the method employed resulted in the separation of the serologically active portion from much inactive material.

[1] Spectroscopic examination performed by Dr. G. I. Lavin.

[2] Chemical analyses carried out by Dr. R. J. Dubos.

However, when partially purified, the antigen or antigens obtained from serum are relatively unstable, its activity decreasing under conditions of storage.

The soluble antigen from a filtered extract of infected skin has been refined in the following manner.

1500 cc. of a filtrate of infected skin were adjusted to pH 4.0 by the addition of 150 cc. of normal HCl. The solution became cloudy and was allowed to stand overnight at 0°C. A dark brown insoluble material was removed by centrifugation and discarded. 1540 cc. of clear reddish brown supernatant material were adjusted to pH 7.0 and an equal volume of a saturated solution of ammonium sulfate was added. A heavy precipitate formed immediately and was removed by centrifugation after storage overnight at +5°C. After dialysis the albumin fraction was found to be serologically inactive and was discarded. The globulin fraction was dissolved in dilute phosphate buffer, pH 7.2, and dialyzed against running water overnight. A small amount of insoluble material which was present after dialysis was removed by centrifugation and discarded. The 525 cc. of soluble dialysate were then dried from the frozen state. The desiccated material dissolved completely in 50 cc. of physiological saline solution. The solution was adjusted to pH 4.5 and a saturated solution of ammonium sulfate in amount sufficient to yield a concentration of 30 per cent was added. The precipitate which formed was removed after storage for 18 hours at 0°C. and dissolved in water. After dialysis against running water the resultant solution was clear and dark reddish brown in color. When diluted 1:128, it formed a precipitate with immune serum, a 4-fold increase in the titer, whereas the volume of the final preparation was 1/12 that of the original filtrate. After the partially purified solution had been stored for a short time at 0°C., insoluble material was observed to have formed. This was removed but more formed. However, removal of the sediment caused no appreciable decrease in the titer of this preparation and after standing for 2 months it still gave a precipitin test in a dilution of 1:128.

A method of purification of soluble antigen in filtered extract of infected skin similar to that employed for purification of antigen in filtered serum seems to be effective in separating the serologically active material from some of the associated inactive substances. However, the final preparations of filtrates of skin which we have obtained have always retained some of the dark color of the original fluids, and from this, as well as from the spontaneous precipitation of material without decrease in titer, it is obvious that they are impure. The partially purified antigen from preparations of infected

skin seems to withstand storage better than that in the apparently cleaner preparations from serum.

Serological and Immunological Studies on Rabbits Injected with Partially Purified Antigen from Extract of Infected Skin

Further study of the nature of the soluble substance of myxoma has included an attempt to determine its antigenicity. Antigen from extracts of infected skin, partially purified without tryptic digestion in the manner described above, was injected into normal rabbits and the response of the animals was investigated.

A sample of normal serum was obtained from each of six young adult male rabbits of the New Zealand breed. The animals were then inoculated intraperitoneally with 2 cc. of a preparation from extracted skin which was known to be non-infectious. The solution had been rendered bacteriologically sterile by Seitz filtration after purification. No untoward reaction developed and during a subsequent 2 week period each animal received a total of 21 cc. of the extract given in six doses. On the 4th day following the last inoculation 10 cc. of blood were taken from each of the rabbits for serological tests. Each of the sera, after inactivation at 56°C. for 30 minutes, gave a precipitin reaction when mixed with the antigen used for immunization. In this instance, dilutions of antisera were tested against constant amounts of antigen; the antibody titers varied between 1:8 and 1:32, but in the majority of the sera they were 1:16. Since precipitins were present in the blood of the rabbits after this short course of treatment, injections were discontinued. 50 cc. of blood were withdrawn from each animal on the 5th day following the last inoculation and the specimens of serum were pooled.

Precipitin Reactions with Anti-Soluble Substance Serum.—The pooled antiserum reacted with partially purified antigen prepared from filtered serum and from extracted skin and also with antigen present in crude preparations of serum, extract of skin, and dermal filtrate. Table VI presents data on such precipitin titrations; the dermal filtrate and filtrate of serum were crude preparations but the antigen from skin had been partially purified. For comparison, the results of titrations with the same antigenic solutions and fibromyxoma serum are included in the table. The pooled anti-soluble substance serum failed to give a precipitate when mixed with any of these serologically active solutions that had been heated at 56°C. for 1 hour; moreover, it did not react with vaccinal dermal filtrate known to con-

tain the heat-labile and heat-stable antigens of vaccinia. Thus, the sera appeared to react specifically with the soluble substance of myxoma. Further evidence for the specificity of the reaction was obtained by absorption experiments.

4 to 6 cc. amounts of pooled anti-soluble substance serum were absorbed with crude dermal filtrate, crude filtrate of serum, and refined extract of infected skin, respectively. The ratio of optimal precipitation was initially determined for each antigenic solution by the method of Dean and Webb. Antiserum and antigen were mixed in the proper amounts, incubated at 56°C. for 1 hour and

TABLE VI

Results of Reaction of Fibromyxoma Serum and Anti-Soluble Substance Serum with the Soluble Antigen

Serum	Antigen	Dilution of antigen						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
Anti-soluble substance	Dermal filtrate	++++	++++	++++	+	−	−	
Fibromyxoma		++++	++++	+	±	−	−	
Anti-soluble substance	Filtrate of serum	++++	++++	++++	++++	++	−	
Fibromyxoma		±	++++	++++	++++	±	−	
Anti-soluble substance	Extract of skin	++++	++++	++++	++++	++++	++	−
Fibromyxoma		±	+++	++++	++++	±	−	−

All sera used in dilution 1:8.
+'s indicate degree of precipitation.

then overnight at 0°C. Precipitate which formed was removed by centrifugation at 0°C. In several different experiments a single absorption with dermal filtrate or extract of skin was sufficient to remove all or practically all the antibody capable of reacting with the antigen employed for absorption. This was not the case in two experiments when filtrate of serum served as the absorbing antigen; here it was necessary to repeat the process two or three times. Absorbed sera were brought to a dilution of 1:8 of the original serum prior to testing for precipitins.

The results of the experiment presented in Table VII show that absorption of anti-soluble substance serum with dermal filtrate completely removes the antibodies that precipitate in the presence of der-

TABLE VII

Results of Absorption Experiments

Anti-soluble substance serum	Antigen	Dilution of antigen						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
Unabsorbed	Dermal filtrate	+++	+++	–	–	–		
	Purified antigen	++	+++	++++	++++	++	++	+
	Filtrate of serum	+++	++++	++	++++	++++	++++	+
	" " " heated	–	–	–	–	–	–	–
Absorbed with dermal filtrate	Dermal filtrate	–	–	–	–	–		
	Purified antigen	–	–	–	–	–	–	–
	Filtrate of serum	+++	++	+++	+	+	–	–
	" " " heated	–	–	–	–	–	–	–
Absorbed with purified antigen	Dermal filtrate	–	–	–	–	–		
	Purified antigen	–	–	–	–	–	–	–
	Filtrate of serum	++++	++++	+++	+	+	–	–
	" " " heated	–	–	–	–	–	–	–
Absorbed with filtrate of serum	Dermal filtrate	–	–	–	–	–		
	Purified antigen	–	–	–	–	–	–	–
	Filtrate of serum	–	–	–	–	–	–	–
	" " " heated	–	–	–	–	–	–	–

All sera used in dilution 1:8.
+'s indicate degree of precipitation.
Purified antigen was obtained from extract of skin.
Heated filtrate of serum was held at 56°C. for 1 hour.
The same antigenic solutions were used throughout.

mal filtrate as well as those that react with extract of skin. The same result is obtained by absorption with extract of skin. These absorbed sera, however, still precipitate when mixed with filtrate of serum; moreover, the amount of residual precipitin that reacts with filtrate of serum is appreciable, for its titer is approximately one-fourth that of the unabsorbed serum. As stated above, repeated absorption of anti-soluble substance serum with filtrate of serum was necessary to remove all of the substances that precipitated in the presence of this antigenic solution. Each absorption with filtrate of serum affected the antibodies that precipitated with dermal filtrate and extract of skin to a greater extent than it did those that precipitated with the absorbing antigen. As a result one or two additions of filtrate of serum sufficed to clear the anti-soluble substance serum of precipitins for dermal filtrate or extract of skin but left behind an appreciable amount of antibody that precipitated with filtrate of serum. This previously unrecognized precipitable material encountered in filtrate of serum is also inactivated by heating at 56°C. for 1 hour (Table VII).

The origin and significance of the second precipitable substance, which occurs in relatively large amounts in filtrates of serum and which differs serologically from the soluble antigen demonstrable in all of the three types of filtrates used in this work, has not yet been determined. Whether it is an entirely independent soluble antigen or a substance closely related to the more common soluble substance remains to be seen. It has been found that purification of the common soluble antigen from serum removes or inactivates the second precipitable substance. For example, a sample of serum which in the crude state reacted with anti-soluble substance serum, either unabsorbed or absorbed with dermal filtrate or antigen from skin, after purification reacted only with unabsorbed serum. It should be noted, however, that antibodies against the second precipitable material were induced by injections of partially purified extract of skin, although absorption with similar extracts of skin did not readily remove these antibodies.

Agglutination Reactions with Anti-Soluble Substance Serum.—Antiserum prepared against purified antigen from extract of infected skin agglutinated certain suspensions of elementary bodies of myxoma. The titer of this serum was generally the same as that of the fibro-

myxoma serum used throughout these studies; a myxoma convalescent serum was slightly more potent than either of the other two. Dermal filtrate obtained during the process of purification of these suspensions of agglutinable elementary bodies contained appreciable amounts of precipitinogen that reacted with anti-soluble substance serum and with fibromyxoma serum. Other suspensions of elementary bodies gave lower agglutinin titers with anti-soluble substance serum than with fibromyxoma serum. Indeed, one suspension was not at all agglutinated by anti-soluble substance serum but reacted with fibromyxoma serum diluted 1:128, and it is of interest to note that dermal filtrate obtained from the rabbits furnishing the suspension of elementary bodies failed to precipitate with anti-soluble substance serum and with fibromyxoma serum.

These observations suggest that the soluble substance of myxoma is only one of two or more antigens involved in the agglutination of elementary bodies by immune serum. Confirmation of this was obtained by the use of suspensions of elementary bodies that had been heated at 56°C. for 1 hour. The agglutinin titer of fibromyxoma and myxoma immune serum was usually as high when tested against heated virus as it was when unheated suspensions were used. The character of the agglutination, however, was different; unheated elementary bodies formed large flocks while heated elementary bodies often gave rise to a very fine granular type of agglutination. On the other hand, suspensions of elementary bodies that were agglutinated by anti-soluble substance serum were, after heating, much less agglutinable in the presence of this serum. The variability in the agglutination of different preparations of elementary bodies is not predictable and will be investigated further. However, results so far obtained are adequate for the following statements: elementary bodies of myxoma, separated from crude dermal pulp containing demonstrable amounts of soluble substance, are agglutinated by anti-soluble substance serum and at least some of this agglutination is due to a heat-labile antigen; elementary bodies contain a relatively heat-stable agglutinogen that reacts with antibodies present in fibromyxoma and myxoma immune serum.

Neutralization Tests with Anti-Soluble Substance Serum.—Pooled anti-soluble substance serum used for the precipitin and agglutination

tests described in the preceding sections was tested for the presence of neutralizing antibodies against the virus of myxoma. Equal volumes of diluted virus and undiluted serum were mixed, stored overnight at 0°C., and injected intradermally into each of two rabbits. Suspensions of myxomatous tissue were infective in dilutions of 10^{-5} or 10^{-6}, in different experiments, when mixed with normal or with anti-soluble substance serum. Fibromyxoma serum regularly neutralized 100 infective doses of the virus. These experiments indicate that antibodies against the soluble substance or substances of myxoma are not effective in neutralizing the virus.

Response to Infection by Rabbits That Had Received Injections of Soluble Antigen.—Following the course of injections of partially purified antigen from infected skin, groups of two rabbits each were tested for response to inoculation with the viruses of myxoma, neuromyxoma, and fibroma, respectively. Animals inoculated with the virus of myxomatosis several days after their seventh injection of antigen from skin, developed a typical infection and died on the 8th and 10th days respectively. Several weeks later the four remaining rabbits were given a second course of four injections of partially purified antigen from skin, because the precipitins in their sera had diminished. 5 days after the last injection in this series, precipitin titers in their sera were again equal to or greater than those induced by the first course of treatments. These animals, inoculated with the viruses of neuromyxoma and fibroma, responded in a manner similar to that of normal control animals. Thus, it appears that rabbits injected with non-infectious extract of myxomatous skin were not rendered immune to the virus of myxoma, neuromyxoma, or fibroma, even though their serum contained precipitins against the soluble substance of myxoma and agglutinins for elementary bodies of myxoma.

DISCUSSION

The experiments have shown the soluble antigen of myxoma present in infected skin and in serum of acutely ill rabbits to be a protein. Rabbits receiving intraperitoneal injections of the partially purified antigen from extracts of infected skin readily develop in their sera specific precipitins against the soluble substance. None of the previously studied soluble antigens associated with virus diseases appears

capable, upon injection into animals, of inducing significant resistance to infection or the formation of appreciable amounts of neutralizing antibodies (yellow fever (3), influenza (8), vaccinia (9, 10)); the soluble substance of myxoma behaves in a similar manner.

Anti-soluble substance serum agglutinates elementary bodies of myxoma under certain conditions. However, the soluble substance is only one of several antigens that take part in the agglutination of elementary bodies of myxoma produced by the serum of animals immune to myxoma; at least one other agglutinogen, relatively heat-stable, is present in or on the virus particles. Thus, in respect to multiplicity of antigens, the elementary bodies of myxoma resemble those of vaccinia (11).

The occurrence of agglutinins for elementary bodies of myxoma in the sera of rabbits recovered from fibroma has been considered by Ledingham (12) to be a possible explanation for the ability of these rabbits to survive infection with the virus of myxoma. The present experiments suggest that the rôle of agglutination alone may be a minor one in this phenomenon since rabbits which possessed agglutinins as the result of injections of soluble antigen, succumbed to infection with the virus of myxoma.

A soluble antigen occurs in the serum of animals ill of yellow fever (3), but it is found in the albumin fraction, while that in the serum of myxomatous rabbits accompanies the globulin fraction.

The present experiments suggest that a second soluble specific substance can be identified in materials from myxomatous rabbits. This substance, also heat-labile, differs serologically from the soluble antigen common to dermal filtrate, extract of skin, and serum of acutely ill animals. The relationship of the two soluble substances remains to be determined.

SUMMARY AND CONCLUSIONS

The soluble antigen of myxoma is a heat-labile protein which has an isoelectric point near pH 4.5 and is precipitated from half saturated solutions of ammonium sulfate. It can be partially purified by methods of differential precipitation based on variations in the pH and electrolyte concentration.

Rabbits receiving the labile, soluble substance of myxoma develop

homologous precipitins and their serum agglutinates elementary bodies of myxoma, provided the dermal pulp from which the bodies are obtained contains the soluble substance; neutralizing antibodies do not appear, however, and the animals are not resistant to infection with the virus of myxoma.

Elementary bodies of myxoma appear to have a heat-stable agglutinogen that operates when brought in contact with serum from animals recovered from myxoma, but little, if at all, when in contact with anti-soluble substance serum.

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