INFECTIOUS MYXOMATOSIS OF RABBITS

II. Demonstration of a Second Soluble Antigen Associated with the Disease

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An earlier communication which dealt with a soluble antigen of infectious myxomatosis (1), contained data indicating the presence of a second serologically active substance in the lesions and in the serum of animals acutely ill with this disease. The observations reported at this time augment the earlier findings and indicate that two serologically distinct soluble antigens are associated with infectious myxomatosis.

Materials and Methods

Source of Soluble Antigens.—Two kinds of extracts of serologically active soluble substances have been used. One, filtered extract of infected skin, is prepared from the skin lesions produced in rabbits by the intradermal inoculation of infective material. The lesions are removed when fully developed but not necrotic, usually on the 6th day after inoculation, macerated in a meat grinder and stored at 0°C. in physiological salt solution. 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 which effectively removes the virus. The second source of soluble antigens is blood drawn by cardiac puncture from rabbits on the 5th or 6th day after the inception of an extensive infection of the skin; the serum is separated and filtered through a Seitz pad.

Preparation of Partially Purified Soluble Antigen (Fraction A).—In a previous report, the partial purification of a soluble antigen associated with myxoma has been described (1). The procedure involves precipitation of virus-free filtrates of myxomatous material in 50 per cent saturated solutions of ammonium sulfate. The precipitate, which contains the active principle, is dissolved in water and freed of ammonium sulfate by dialysis overnight against running water. The solution is then brought to pH 4.5 and ammonium sulfate is added to a concentration of 30 per cent. The precipitate which is obtained is dissolved and dialyzed against water. This solution constitutes fraction A.

Immune Sera.—Fibromyxoma serum was collected from rabbits which had been repeatedly inoculated with myxoma virus after recovery from an infection with fibroma virus. Fibroma serum was obtained from rabbits a month after inoculation with fibroma virus. Myxoma serum was collected from rabbits which had survived an attack of myxomatosis; two samples were available, one of which was supplied by Dr. R. F. Parker. Anti-soluble substance serum 9212, a pooled serum, was obtained

from rabbits following the injection of a partially purified extract (fraction A) of skin. These various sera were used in work previously reported (1). Sera from normal rabbits or from rabbits recovered from vaccinal infections were used to control the specificity of reactions.

Precipitin Reactions.—As a rule, graded dilutions of the antigen to be tested were mixed with constant amounts of immune sera; occasionally, however, graded dilutions of antisera were added to constant amounts of antigen. All test mixtures were incubated in closed racks overnight at 50°C.

EXPERIMENTAL

The previous experiments (1) that had suggested the presence of a second specific soluble substance in infectious myxomatosis were made with pooled antiserum which had been prepared in rabbits by the injection of partially purified virus-free material (fraction A) from myxomatous skin lesions. This antiserum, 9212, after absorption with fraction A no longer reacted with this antigen, but still precipitated with serum from rabbits moribund with infectious myxomatosis. For the sake of simplicity, the soluble antigen which has already been described in considerable detail (1) will henceforth be referred to as antigen A; the soluble substance which reacted with the antiserum absorbed free of A antibodies will be designated antigen B.

Two observations on antigen B were recorded in the studies on the characteristics of the first soluble antigen (1), and, since both have been confirmed, they may be briefly summarized. Antigen B, like antigen A, is heat labile for, after being heated at 56°C. for ½ hour, it no longer precipitates with its specific antibody. Furthermore, antigen B is discarded or inactivated during the procedure employed for the purification of antigen A.

Separation of the Two Soluble Antigens

It seemed logical to determine at what stage antigen B was lost in process of purifying antigen A. Moreover, it was obviously desirable to obtain solutions of each antigen uncontaminated by the other. Accordingly, crude materials containing the soluble substances were subjected to the method of fractionation previously employed. The presence or absence of the two soluble antigens in material obtained at each step in the fractionation was established by two sets of precipitin titrations. One test was made with antiserum 9212, which contained both A and B antibodies, and the other with antiserum 9212 which had been absorbed free of A antibodies. A representative experiment is detailed below.

42 cc. of filtered serum obtained from a rabbit moribund with myxoma were mixed with 42 cc. of a saturated solution of ammonium sulfate. The white precipitate which

formed was removed by centrifugation, dissolved in dilute phosphate buffer and dialyzed overnight against running water. The supernatant solution, containing material not precipitated in a concentration of 50 per cent ammonium sulfate, was dialyzed in a similar manner and each dialysate, after addition of NaCl to physiological concentration, was tested for precipitinogens with antiserum 9212 which contained A and B antibodies, and with another portion of this antiserum which had been absorbed free of A antibodies. The globulin fraction contained both precipitable substances; the solution of albumin contained only a negligible amount of serologically active material. Further fractionation of the globulin was carried out as follows: the dialysate was brought to pH 4.5 by the addition of N HCl, and a sufficient amount of a saturated solution of ammonium sulfate was added to bring the final concentration to 30 per cent saturation. The precipitate which formed was removed by centrifugation, dissolved in dilute buffer and dialyzed against running water overnight. The supernatant fluid was neutralized and enough of a saturated solution of ammonium sulfate was added to make a final concentration of 50 per cent saturation. The precipitate which formed was removed by centrifugation, dissolved in dilute buffer and dialyzed. The material precipitated at 30 per cent saturation with ammonium sulfate and that precipitated between 30 and 50 per cent saturation were about equal in amount and similar in appearance. Precipitin tests were performed with solutions of these fractions after the NaCl concentration had been brought to 0.85 per cent.

The data provided in the protocol and summarized in Table I indicate that both antigens were present in the fraction of the serum filtrate that was insoluble in a 50 per cent saturated solution of ammonium sulfate. Further fractionation of the globulin by precipitation in a 30 per cent saturated solution of ammonium sulfate at pH 4.5 brought about a separation of the two antigens: antigen A was insoluble in this concentration of the electrolyte while antigen B remained in solution and was subsequently precipitated by raising the concentration of ammonium sulfate to 50 per cent saturation. Similar results were obtained when the same type of procedure was applied to a filtrate of crude extract of myxomatous skin which was known to contain both antigens.

Immunization of Rabbits with Partially Purified and Whole Extracts of Myxomatous Skin and with Whole Serum from Acutely Ill Rabbits

According to the results of precipitin tests, a single fractionation with ammonium sulfate generally resulted in a sharp separation of antigens A and B, yet the employment of this method apparently left the preparation of antigen A contaminated with immunologically significant amounts of antigen B. It has already been pointed out that antiserum 9212, obtained by injecting rabbits with fraction A of an extract of myxomatous skin, contained antibodies against both types of soluble antigen. Hence, the injected material may be assumed to have contained antigen B although this substance was unrecognized at the time and therefore not sought for.

At the beginning of the present work, before the identity of antigen B appeared well established, it seemed desirable to determine whether antisera similar to 9212 could be regularly obtained. Accordingly, rabbits were injected with preparations apparently free of antigen B and with preparations containing both antigens A and B. Four rabbits were immunized in a manner similar to that employed in a previous experiment (1), that is, by means of a series of injections of partially purified extract

TABLE I

Precipitin Reactions of Fractions of Serum Antigen

Fraction of serum antigen	Immune serum 1:8	Dilution of antigen						
		Undiluted	1:2	1:4	1:8	1:16	1:32	1:64
Total albumin	9212 (anti A and B)	−	−	−	−	−	−	−
	9212 (absorbed with A)	−	−	−	−	−	−	−
Total globulin	9212 (anti A and B)		++++	++++	++++	+++	±	−
	9212 (absorbed with A)		++++	++++	++++	+	−	−
Globulin insoluble in 30 per cent ammonium sulfate at pH 4.5 (fraction A)	Normal	−	−	−	−	−	−	−
	Fibromyxoma	+	±	−	++++	+++	++	±
	9212 (anti A and B)	+++	++++	++++	++++	+++	+	−
	9212 (absorbed with A)	++	−	−	−	−	−	−
Globulin insoluble in 50 per cent ammonium sulfate at pH 7.0 (fraction B)	Normal	−	−	−	−	−	−	−
	Fibromyxoma	++	−	−	−	−	−	−
	9212 (anti A and B)	++++	++++	++++	++++	−	−	−
	9212 (absorbed with A)	+++	++++	++++	−	−	−	−

+'s indicate degree of precipitation.

(fraction A) of myxomatous skin. This immunizing material was obtained in the manner described and was shown to possess a high titer when tested for precipitinogens with antiserum 9212 (anti-soluble substance serum containing A and B antibodies); moreover, the material injected into the rabbits was shown to absorb from antiserum 9212 all antibodies against A and to leave those against B. Two additional groups of 4 rabbits each were immunized; one group received whole filtered extract of myxomatous skin, while the other was given whole filtered serum from rabbits moribund with myxomatosis. Both these immunizing materials were known to contain antigens A and B. The rabbits were bled at intervals and their sera tested

TABLE II

Precipitin Reactions with Antigens A and B and Sera from Rabbits Immunized with Three Preparations of Soluble Antigen

Immune serum	Material used for immunization	Test antigen	Dilution of antiserum						Test antigen	Dilution of antiserum					
			Undiluted	1:2	1:4	1:8	1:16	1:32		Undiluted	1:2	1:4	1:8	1:16	1:32
9212	Fraction A of skin	Fraction A of skin 1:6	++++	++++	++++	++++	+++	−	Fraction B of skin 1:8	++++	++++	++++	++++	+++	−
68	Whole filtrate of serum (containing antigens A and B)		++++	++++	++++	++++	++++	++++		+++	+	−	−	−	−
69			++++	++++	++++	++++	++++	++++		++++	+++	+++	−	−	−
70			++++	++++	++++	++++	++++	++++		+++	−	−	−	−	−
71			++++	++++	++++	++++	++++	++		±	+	++	−	−	−
74	Whole filtered extract of skin (containing antigens A and B)		++++	++++	++++	++++	++++	+		±	−	−	−	−	−
75			++++	++++	++++	++++	+++	±		++++	++++	++++	++++	+	−
76	Fraction A of skin		++	+++	++++	++	−	−		+++	++++	++	−	−	−
77			±	+++	+++	+++	++	−		++++	++++	++++	±	−	−
78			++++	++++	++++	++++	+++	++		++++	++++	++++	+++	−	−
79			++++	++++	++++	+++	++++	+++		++++	++++	++	±	−	−

+'s indicate degree of precipitation.

for the presence of precipitating antibodies against antigens A and B. The titers obtained at the end of the period of injection are recorded in Table II.

Examination of Table II reveals that the 4 rabbits (68–71) injected with whole filtered serum produced antibodies in high titer against antigen A but in much lower concentration for antigen B. Furthermore, of the 4 rabbits receiving extract of whole filtered skin only 2 (74, 75) developed antibodies against either of the antigens in concentration high enough to be detected and only 1 (75) possessed antibodies against B. On the other hand, the injection of fraction A, which contained no demonstrable antigen B, induced the development of both A and B antibodies in the rabbits receiving it (76–79). This finding confirms our previous experience in the production of antiserum 9212. Moreover, sera 76–79, when absorbed with partially purified extract of skin (fraction A), behaved in a manner similar to antiserum 9212, *i.e.*, they still precipitated with whole serum which contained both antigens A and B and with fraction B of skin and serum.

Inhibition of Antibodies by Non-Precipitable Forms of Antigens A and B

The relative instability of partially purified antigen A, even when dried from the frozen state and stored in the cold, has been noted (1) in preparations tested for precipitinogens. It seemed possible that slightly changed forms of the soluble antigen might still combine with their specific antibodies although visible precipitation did not occur. The ability of non-precipitable forms of the antigens to inhibit their antibodies was investigated in the manner outlined below.

An active fraction A of skin antigen which had been heated at 56°C. for 1 hour and which no longer precipitated with antiserum 9212 was added to an equal volume of this serum which contained antibodies against A and B. The mixture was incubated at 56°C. for 1 hour and stored overnight at 5°C. No precipitation occurred and the treated antiserum was tested for its activity with antigens A and B. In a similar manner an active fraction B of skin antigen, heated at 56°C. for 1 hour, was found to give no precipitate when mixed with antiserum 78 known to contain A and B antibodies. Equal parts of the heated antigen and the antiserum 78 were mixed, incubated at 56°C. for 1 hour, stored at 5°C. overnight and then tested for precipitating antibodies with antigens A and B. The results of the titrations are presented in Table III.

It is apparent from the results presented in Table III that the solutions of the two antigens which had completely lost their power to precipitate with antibodies as a result of heating at 56°C. were capable of specifically inhibiting their respective antibodies. Similar tests carried out with materials from rabbits infected with vaccine virus did not result in inhibition of antibodies against either antigen A or B of myxomatosis. Furthermore,

heated preparations of myxoma antigens did not inhibit the precipitin reactions of vaccinia. Specific inhibition of antivaccinal antibodies has been demonstrated, however, with degraded soluble antigens of vaccinia (2).

Occurrence of Antigen B in Infected Animals

The observations recorded indicate that two soluble antigens can be identified in myxomatous tissue of rabbits and in the sera of animals during the height of the disease. There seemed little reason to believe that the

TABLE III

Precipitin Reactions of Anti-Soluble Substance Sera after Treatment with Non-Precipitable Antigen

Immune serum	Test antigen	Dilution of antigen					
		1:2	1:4	1:8	1:16	1:32	1:64
9212 untreated (anti A and B)	Fraction A	++	+++	++	++	++	−
	Fraction B	++++	++++	++++	++	−	−
9212 treated with non-precipitable A	Fraction A	−	−	−	−	−	−
	Fraction B	++++	+++	++	+	−	−
		Dilution of antiserum					
		1:2	1:4	1:8	1:16	1:32	1:64
78 untreated (anti A and B)	Fraction A	++++	++++	++++	+++	+	−
	Fraction B	++++	++++	++++	−	−	−
78 treated with non-precipitable B	Fraction A		++++	++++	++++	−	−
	Fraction B		−	−	−	−	−

+'s indicate degree of precipitation.

second antigenic substance was formed during the process of manipulation incident to purification of these substances; nevertheless, this possibility had to be considered. Two types of evidence were obtained which demonstrated the occurrence of antigen B in the natural infection.

The first kind of evidence consisted of the detection of antigen B in the serum of rabbits acutely ill with myxomatosis. In earlier work, at a time when antigen B had not been recognized, it appeared that the first demonstrated specific soluble substance associated with myxoma (antigen A) was sometimes absent from samples of sera taken from moribund animals or was present in low concentration. Such sera, which contained no demon-

strable antigen A, were often found to contain large amounts of precipitable substance (antigen B) when tested with antisera known to precipitate with

TABLE IV

Titer of Precipitinogens (A and B) in Whole Filtrates of Myxomatous Serum Tested with Fibromyxoma Serum and Anti-Soluble Substance Serum 78

Fresh filtrate of serum	Immune serum 1:8	Dilution of antigen							Remarks
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	
813	Fibromyxoma	+	±	+++	++++	++++	+++	±	Antigen A present
	78	+	++++	++++	++++	++++	++++	++	
649	Fibromyxoma	–	–	–	–	–	–	–	Only antigen B present
	78	++++	++++	++++	+++	++	–	–	
1	Fibromyxoma	–	–	–	–	–	–	–	Only antigen B present
	78	+++	++++	++++	++++	–	–	–	
653	Fibromyxoma	–	–	–	–	–	–	–	Only antigen B present
	78	++	++++	+++	++	+	–	–	

+'s indicate degree of precipitation.

Fibromyxoma serum gives precipitates only with antigen A, while anti-soluble substance serum 78 reacts with antigens A and B.

TABLE V

Precipitin Reactions of Two Samples of Myxoma Serum and Two Samples of Fibroma Serum with Antigen A and Antigen B

Antigen	Immune serum	Dilution of antigen					
		Undiluted	1:2	1:4	1:8	1:16	1:32
Fraction A	Myxoma 1	++++	++++	++++	++++	++	–
	Myxoma 2	++++	++++	++++	++++	++++	+
	Fibroma 1	++++	++++	++++	+	–	–
	Fibroma 2	++++	++++	++++	++	–	–
Fraction B	Myxoma 1	++++	+++	+	±	–	–
	Myxoma 2	++	–	–	–	–	–
	Fibroma 1	++++	+++	+	–	–	–
	Fibroma 2	±	–	–	–	–	–

+'s indicate degree of precipitation.

both A and B. These sera received no treatment except filtration through Seitz pads, hence the formation of antigen B by denaturation of a native substance in the serum seemed unlikely. The results of precipitin titrations obtained with sera containing both antigens A and B, and with others containing only antigen B are illustrated in Table IV.

The second type of evidence was furnished by the detection of B antibodies in the sera of two animals following recovery from infectious myxomatosis. It is of interest to note that B antibodies were also demonstrated in the serum of one of two rabbits convalescent from fibromatosis. The results of titrations with sera from these 4 animals are presented in Table V. The occurrence of B antibodies in convalescent animals may be taken as evidence that antigen B is present during the acute infection.

DISCUSSION

Infected tissue and serum from rabbits moribund with myxomatosis apparently contain two specific soluble antigens which have been designated A and B. Both antigens are inactivated at 56°C. and both are recoverable with the globulin fraction of solutions containing them. Antigen A is insoluble in a 30 per cent saturated solution of ammonium sulfate at pH 4.5, while antigen B is soluble in this concentration but precipitates in a half saturated solution of the salt. Both antigens readily lose their ability to flocculate in the presence of their specific antibodies. Nevertheless, nonprecipitable forms of both antigens are capable of inhibiting their specific antibodies. The sera of certain rabbits convalescent from infection with fibromatosis and myxomatosis contain antibodies against both antigens.

The fibromyxoma serum used throughout an earlier series of experiments (1) contained adequate amounts of A antibodies, but, in the dilutions employed, failed to react appreciably with antigen B. This latter fact undoubtedly facilitated the studies dealing with the characteristics of antigen A and with the development of methods for its partial purification. On the other hand, this lack of B antibodies in the fibromyxoma serum delayed the identification of antigen B.

Antigens A and B are immunologically distinct and apparently can be separated by simple physical means; nevertheless, it has been observed that rabbits injected with preparations of antigen A regularly develop both A and B antibodies. Indeed, such a procedure more uniformly induces the production of B antibodies than does immunization with crude materials rich in both serologically active substances. An explanation of these phenomena is not at hand. One point is obvious, however, that is the lack of purity of preparations of antigen A. These undoubtedly contain antigen B in amounts which may be comparatively small but are still immunologically significant. If antigen B in small amounts is capable of eliciting antibody formation, then why does immunization with crude preparations of skin or serum containing large amounts of this substance fail to call

forth B antibodies? Only conjectures can be offered; one of the more obvious is that some one or more of the extraneous substances in the crude preparations interferes with the ability of antigen B to stimulate antibody production.

SUMMARY

A second soluble antigen, separable from the virus, occurs in extracts of infected skin and in the serum of rabbits acutely ill with infectious myxomatosis. Like the first antigen (A), the second (B) is heat labile and has certain characteristics of a globulin. The two antigens precipitate in different concentrations of ammonium sulfate and can be separated by this method. Neither of the antigens after being heated at 56°C. precipitates in the presence of specific antibody but each is capable of inhibiting the activity of its antibody.

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