STUDIES ON THE SOLUBLE PRECIPITABLE SUBSTANCES OF VACCINIA

III. The Precipitin Responses of Rabbits to the LS Antigen of Vaccinia

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In previous papers, Craigie and Wishart (1934 a; 1936 a, b) reported that the soluble precipitable substances of vaccinia and the corresponding agglutinogens of the elementary bodies have a complex antigenic composition. Two antigenic components have been differentiated and these show a marked difference in their thermostability. These have been termed the L (labile) and S (stable) components, respectively. The former is labile at 56°C. and the latter is stable at temperatures up to 100°C. Absorption tests have shown that each component reacts with its homologous antibody, whether present in solution or fixed to the elementary body, and that the L and S antibodies are distinct and may be obtained separately. The L and S antigens, on the other hand, behave *in vitro* as if components of a complex LS antigen.

The *in vitro* analysis of the antigens of vaccinia has been accompanied by extensive observations on the precipitin and agglutinin responses of rabbits to the injection of elementary bodies and the soluble antigens of vaccinia. These observations will be summarized in this paper. Since the agglutinin or precipitin response of over a hundred rabbits to hyperimmunization has been followed it is possible to give only representative examples, indicating at the same time the extent of variations in response which have been encountered. This paper also supplements the two previous papers in this series in providing further information regarding the L and S precipitin sera used in the investigation of the soluble precipitable substances of vaccinia.

EXPERIMENTAL

Methods

The vaccine virus preparations used have been chiefly obtained with the C.L. strain of lapine (Craigie and Wishart, 1934 a). All data given in tabular form in this paper relate to this strain. Where details of methods are omitted they will be found in papers to which reference is given. All sera, following their separation from the blood clot, were heated for 45 minutes at 56°C. as a precaution against the antagonistic effect on precipitation demonstrable with low titre precipitin sera (Craigie, 1932). Agglutinins were titrated by the method described by Craigie and Wishart (1934 a), and the complement-fixing antibodies by the method described by the same authors (1934 b). Suitable dilutions of Seitz filtrates of lapine or LS fraction (Craigie and Wishart, 1936 b) were used for the titration of precipitins, the tests being incubated for 18 hours at 50°C.

Filtrate or LS fraction heated for 1 hour at 70°C. was used for the direct titration of S antibody, while unheated antigen was used to obtain the titre of the predominant antibody. Both the LS and S antigens are precipitated by S antibody, and an LS titre which does not definitely exceed the S titre offers no clue to the L antibody titre. Before the L titre can be determined the S antibody must be removed from the serum by the application of an equivalent amount of S antigen (Craigie and Wishart, 1936 b), and this was resorted to when necessary. On the other hand, it has been found that an LS titre more than twice as great as the S titre may be provisionally accepted as the L titre provided that the test LS and S antigens contain similar amounts of S antigen.

Antibody Formation Following Vaccination

Table I and the first portion of Fig. 1 show the development of agglutinins, precipitins, and complement-fixing antibodies following dermal vaccination. Only unheated elementary bodies and lapine filtrate were used in the serum titrations, and the figure and Table I therefore indicate only the titre of the predominant type of antibody.

The antibody curves shown are typical in respect of the times of first appearance and maximum presence of antibody. The agglutinin, complement-fixing, and precipitin titres, shown in Fig. 1, however, are higher than is usually the case. Following vaccination the maximum agglutinin and complement-fixing titres generally range from 1 in 160 to 1 in 640 while precipitin titres range from 1 in 2.5 to 1 in 20. The precipitins (Fig. 1) show a relative increase in their ratio to the agglutinins from the 7th to the 16th day. This is probably more apparent than real, reflecting the inaccuracies of precipitin tests car-

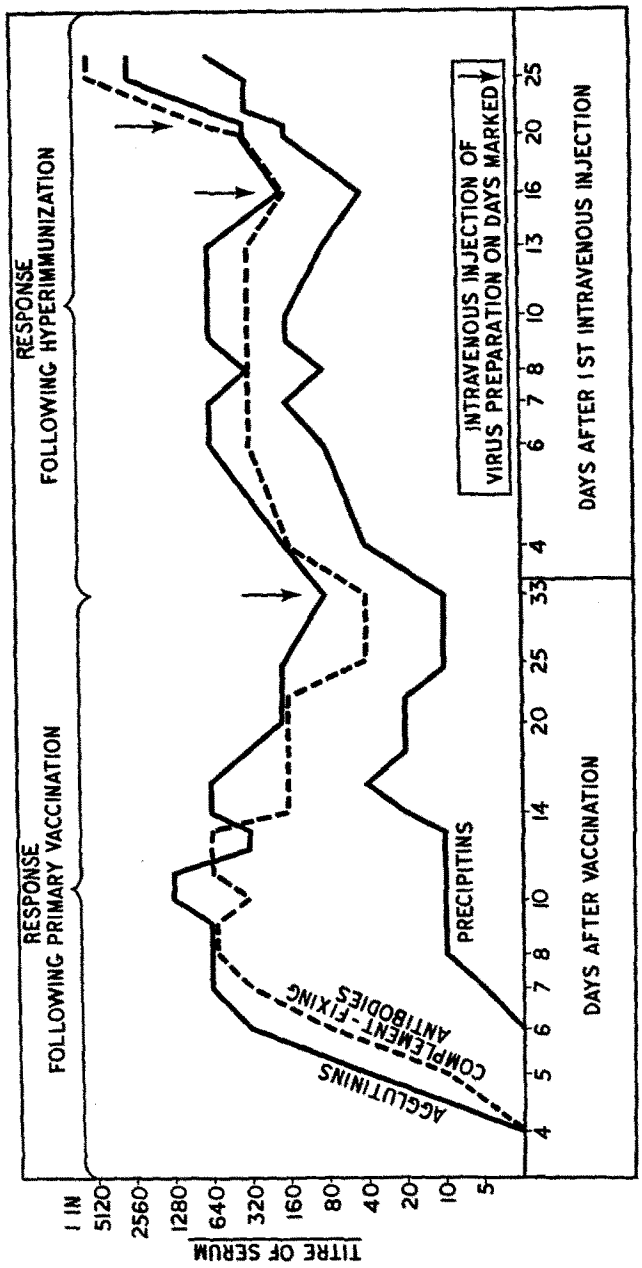

FIG. 1. Antibody response of rabbit 8-5. Virus preparation was a suspension of elementary bodies in lapine Seitz filtrate.

ried out with crude lapine filtrates. Other series of vaccinated rabbits have shown a close parallelism between the precipitins, agglutinins, and complement-fixing antibody, and further observations are required on the antibody response to vaccination, embracing estimations of L and S antibodies with the methods now available. It is known, however, that both L and S antibodies develop after vaccination. As shown in Fig. 1, the antibodies exhibit a progressive decrease in the 3rd week following vaccination and this continues. Rabbits show a

TABLE I

Response to Dermal Vaccination

Rabbit No.	Day after vaccination	Agglutinin titre	Complement-fixing titre	Precipitin titres
7-5	4th	1 in 5	<1 in 20	<1 in 2.5
	7th	1 in 320	1 in 160	1 in 2.5
	10th	1 in 320	1 in 160	1 in 10
	16th	1 in 160	1 in 80	1 in 20
	33rd	1 in 20	1 in 20	1 in 10
9-7	4th	<1 in 5	<1 in 20	<1 in 2.5
	7th	1 in 40	1 in 20	<1 in 2.5
	10th	1 in 320	1 in 320	<1 in 2.5
	16th	1 in 160	1 in 80	1 in 2.5
	33rd	1 in 80	1 in 40	<1 in 2.5
9-9	4th	1 in 40	<1 in 20	<1 in 2.5
	7th	1 in 640	1 in 320	<1 in 2.5
	10th	1 in 320	1 in 160	1 in 5
	16th	1 in 160	1 in 80	1 in 5
	33rd	1 in 40	1 in 20	1 in 5

considerable variation in the amounts of antibodies which persist 1 or 2 months following vaccination. Precipitins generally disappear in 4 to 6 weeks while small amounts of agglutinins may persist for a longer period.

Infective virus is not essential for the formation of antibodies in the normal rabbit, since intravenous injection of both elementary bodies rendered noninfective by heat or formalin and virus-free Seitz filtrates produce agglutinins, complement-fixing antibodies, and precipitins (Craigie, 1934). Certain differences, however, were found in the

responses due to vaccination and to inoculation with noninfective preparations. Reinvestigation with special reference to L and S antibodies will be necessary before the significance of these differences can be assessed.

Antibody Formation on Hyperimmunization

The latter part of Fig. 1 shows that a further development of antibodies occurs when immune rabbits are inoculated with virus preparations and that higher antibody titres may be obtained by hyperimmunization than by vaccination alone. In this instance the agglutinogens and precipitinogens of vaccinia were both injected intravenously, the injections consisting of elementary bodies suspended in a Seitz filtrate of lapine which contained the soluble precipitable substances. This fractionation and subsequent pooling of the antigens was necessary in order to obtain the total antigens of vaccinia in a form suitable for intravenous injection. Apart from variation in degree of antibody response to vaccination, rabbits show a considerable variation as regards the later rate of fall of antibody titre. Reference to the tables in this paper will show that the sera of rabbits, 2 to 3 months after vaccination, contain no demonstrable amounts of precipitin. At this interval after vaccination the agglutinins either show a very low titre or are not demonstrable. Hyperimmunization appears to be equally effective whether 3 weeks or 3 months have elapsed since vaccination, and has been extensively employed in the study of the antigenic qualities of the LS antigen. While it might be objected that this method indicates only the effectiveness of the preparations as secondary stimuli, the use of normal rabbits is open to more serious objection unless very rigid isolation of the animals and extensive testing of the preparations for the complete absence of infective virus is practised. Duran-Reynals (1931) has pointed out that the risk of infection of rabbits with vaccine virus is increased by intravenous injection and the taking of blood samples. We have, on a number of occasions, observed the development of agglutinins for elementary bodies in normal rabbits merely placed in the same room with vaccinated rabbits, but not in proximity to them. This formation of agglutinins is accompanied by the development of immunity, probably as a result

of subclinical infection *via* the respiratory tract. Normal rabbits can, therefore, only be employed in the investigation of antigenic qualities of vaccinia antigens if fallacies due to infection with the virus are excluded by rigid isolation, adequate testing of the inocula for absence of infective virus, and the provision of an adequate number of uninoculated control animals. For this reason we have resorted to hyperimmunization of immune rabbits, permitting a sufficient interval for the disappearance of precipitins to elapse after vaccination.

Hyperimmunization with Heated Elementary Bodies

As previously reported (Craigie and Wishart, 1934 a) vaccination or hyperimmunization with infective elementary bodies leads to the production of L and S agglutinins and, as later found, L and S precipitins also. Heated elementary bodies were found to absorb only the S antibody. Table II provides examples of the increase of S precipitin which results when rabbits are hyperimmunized with elementary bodies heated at 90°C. The responses shown in this table are representative of a larger series.

Samples of serum indicated in Table II were examined by absorption and no L antibody was found. From this and other observations we conclude that exposure to 70°C. for an hour completely inactivates the L antigen while the S antigen remains antigenic after being heated at 90°C. for 1 hour. Although the L antigen is destroyed at 56°C. as far as its reactivity *in vitro* is concerned, it seems preferable to use a temperature of 70°C. to ensure destruction of the last traces of this antigen when the material is to be used for serum production.

Hyperimmunization with Infective Elementary Bodies and Dissociated Antigen

Table III shows the responses of six rabbits to hyperimmunization with infective elementary bodies.

The first injection consisted of antigen dissociated from a washed suspension of elementary bodies and in this experiment only a slight response occurred in three of the rabbits. In other experiments where further injections of dissociated antigen were given, a more definite formation of L and S antibodies has resulted. Compared with LS fraction, dissociated antigen preparations contain considerably less

TABLE II

Responses to Injection of Heated Elementary Bodies

Date of injection	Injection (intravenously)	Date of serum test	Rabbit No. and precipitin titre							
			D3-13		D3-16		D3-22		D3-28	
			LS	S	LS	S	LS	S	LS	S
1935		*1935*								
Mar. 16	Dermal vaccination	Apr. 5	1 in 5	1 in 2.5	0	1 in 5	1 in 20	1 in 5	0	1 in 2.5
Apr. 5	0.5 cc. E.B. 90°C.	Apr. 10	1 in 80	1 in 80	1 in 80	1 in 80	1 in 160	1 in 160	1 in 80	1 in 80
Apr. 10	0.5 cc. E.B. 90°C.	Apr. 15	1 in 80	1 in 160	1 in 160	1 in 160	1 in 160	1 in 160	1 in 160	1 in 160
Apr. 15	0.5 cc. E.B. 90°C.	Apr. 23	1 in 40	1 in 40	1 in 40	1 in 40	1 in 20	1 in 20	1 in 20	1 in 20
May 10	0.75 cc. E.B. 70°C.	May 16	1 in 40	1 in 80	1 in 40	1 in 80	1 in 20	1 in 20	1 in 20	1 in 20
May 16	1.0 cc. E.B. 70°C.	May 28	1 in 80	1 in 160	—	—	—	—	1 in 20	1 in 20

E.B. = elementary body suspension. 0 = titre less than 1 in 2.5. 90°C. = suspension heated at 90°C. for 1 hour. 70°C. = suspension heated at 70°C. for 1 hour.

TABLE III

Response to Injection of Infective Elementary Bodies

Date of injection	Injection (intravenously)	Date of serum test	Rabbit No. and precipitin titre											
			D2-26		D2-31		D2-32		D2-33		D2-38		D2-41	
			LS	S	LS	S	LS	S	LS	S	LS	S	LS	S
1935		*1935*												
Jan. 15	Dermal vaccination	Feb. 8	0	0	1 in 40	1 in 10	1 in 10	0	1 in 5	0	1 in 20	0	0	0
Feb. 8	D.A. 1 cc.	Feb. 15	0	0	1 in 80	1 in 40	1 in 10	1 in 5	1 in 20	1 in 10	1 in 20	0	1 in 20	0
Feb. 15	E.B. 1 cc.	—	—	—	—	—	—	—	—	—	—	—	—	—
Feb. 22	E.B. 1 cc.	Feb. 27	1 in 80	1 in 20	1 in 80	1 in 40	1 in 40	1 in 20	1 in 160	1 in 40	1 in 80	1 in 20	1 in 160	1 in 40
Feb. 27	E.B. 1 cc.	Mar. 5	1 in 160	1 in 40	1 in 320	1 in 80	1 in 80	1 in 20	1 in 320	1 in 80	1 in 160	1 in 40	1 in 320	1 in 160

D.A. = dissociated antigen from washed elementary bodies. E.B. = washed elementary body suspension. Test antigen = LS; Seitz filtrate, S; Seitz filtrate heated at 70°C. 0 = titre less than 1 in 2.5.

precipitable substance (Craigie and Wishart, 1936 a) and we consider that the feeble response to injection of dissociated antigen is referable to its small content of antigen. The subsequent injections in the experiment shown in Table III consisted of infective elementary bodies and these brought about a considerable rise in precipitin titre. It will be noted that the LS titres (*i.e.* for untreated filtrate), in contrast to those produced by heated elementary bodies (Table II) were significantly higher than the S titre (*i.e.* for heated filtrate). Comparison of Table III with Table IV will show that rather higher precipitin titres were produced with infective elementary bodies than with LS antigen. This slightly greater effectiveness of elementary bodies in evoking a response seems to be generally true.

Hyperimmunization with LS Fraction

Seitz filtrates of lapine have been used for the production of precipitin sera and, more recently, the LS fraction (partially purified LS antigen) which is derived from Seitz filtrates (Craigie and Wishart, 1936 b). Tables IV and V show the responses of rabbits to intravenous hyperimmunization with LS fraction and S (*i.e.* heated LS) fraction respectively.

It will be noted that the doses of antigen were graded and it is believed that this procedure, with a 5 to 6 day spacing of the doses, generally evokes the maximum response of which the individual rabbit is capable. The doses are expressed in arbitrary units, one unit representing that amount of LS antigen which will just give perceptible precipitation with the optimum amount of precipitin in a volume of 0.5 cc. The variations in degree of response shown in Tables IV and V may be taken as representative. Occasionally a maximum titre of 1 in 320 has been encountered and on the other hand a few rabbits have failed to attain either an L or S titre greater than 1 in 10. A comparison of Tables IV and V will show that only after injection with unheated LS fraction may the LS titre exceed the S titre. By means of absorption tests it was shown that the rabbits inoculated with unheated LS fraction developed both L and S antibodies, while those inoculated with S fraction developed S antibodies only. The L antigen, therefore, is completely inactivated on heating LS fraction for 1 hour at 70°C.

As shown in Tables IV and V variations occur in the time taken

TABLE IV

Response to Injection of LS Fraction

Date of injection	Injection LS fraction (intravenously)	Date of serum test	Rabbit No. and precipitin titre							
			E9		E1-0		E1-1		E1-2	
			LS	S	LS	S	LS	S	LS	S
1935		*1935*								
July 10 to 11	Dermal vaccination	Oct. 12	0	0	0	0	0	0	0	0
Oct. 12	5 units	Oct. 18	0	0	1 in 5	1 in 5	1 in 10	1 in 5	0	1 in 5
Oct. 18	20 units	Oct. 23	1 in 80	1 in 20	1 in 20	1 in 20	1 in 40	1 in 20	1 in 20	1 in 5
Oct. 23	20 units	Oct. 28	1 in 80	1 in 40	1 in 40	1 in 40	1 in 40	1 in 20	1 in 40	1 in 10
Oct. 29	100 units	Nov. 5	1 in 80	1 in 40	1 in 40	1 in 20	1 in 160	1 in 80	1 in 40	1 in 10
Nov. 6	100 units	Nov. 13	—	—	1 in 40	1 in 20	—	—	1 in 80	1 in 10
Nov. 14	400 units	Nov. 19	—	—	1 in 10	1 in 10	—	—	1 in 10	1 in 5

Test antigens—LS = LS fraction, S = LS fraction heated at 70°C. for 1 hour. 0 = titre less than 1 in 2.5.

TABLE V

Response to Injection of S Fraction

Date of injection	Injection S fraction (intravenously)	Date of serum test	Rabbit No. and precipitin titre							
			E5		E1-4		E1-5		E1-8	
			LS	S	LS	S	LS	S	LS	S
1935		*1935*								
July 9	Dermal vaccination	Oct. 12	0	0	0	0	0	0	0	0
Oct. 12	5 units	Oct. 18	1 in 5	1 in 10	0	1 in 10	0	1 in 5	1 in 5	1 in 10
Oct. 18	20 units	Oct. 23	1 in 40	1 in 80	1 in 20	1 in 40	1 in 10	1 in 20	1 in 40	1 in 80
Oct. 23	20 units	Oct. 28	1 in 80	1 in 160	1 in 40	1 in 40	1 in 10	1 in 20	1 in 40	1 in 80
Oct. 29	100 units	Nov. 5	1 in 80	1 in 80	1 in 40	1 in 40	1 in 20	1 in 20	1 in 80	1 in 80
Nov. 6	100 units	Nov. 13	1 in 80	1 in 80	1 in 40	1 in 40	1 in 20	1 in 20	1 in 40	1 in 40
Nov. 14	400 units	Nov. 19	1 in 40	1 in 40	1 in 20	1 in 20	1 in 10	1 in 20	1 in 20	1 in 20

S fraction = LS fraction heated at 70°C. for 1 hour. Test antigens—LS = LS fraction, S = LS fraction heated at 70°C. for 1 hour. 0 = titre less than 1 in 2.5.

for the maximum titres to be reached. These tables also illustrate a general finding, that after several injections of antigen the ability of the rabbit to respond is impaired and that thereafter the antibody titre falls in spite of an increase in the dose of antigen. A good deal of variation occurs in the ratio of L to S antibody produced by inoculation of LS antigen, and S antibody may predominate. L sera which are necessary for identification of the L antigen are prepared by absorbing out the S antibody present in a serum containing both antibodies. In an endeavour to obtain high titre L sera and facilitate the absorption of S antibody from them, attempts were made to increase the relative proportion of L antibody by adding an excess of S serum to LS fraction prior to injecting it. While this procedure does not necessarily completely prevent formation of S antibody the results obtained certainly suggest that it is of value in generally decreasing the S response without affecting the development of L antibody.

In general, elementary body suspensions are rather more effective than LS fraction in promoting L precipitin formation. However, in view of evidence that infective elementary bodies possess other agglutinogens as well as the LS antigen (Craigie and Wishart, 1936 b), the serological behaviour of sera produced by their inoculation should be interpreted with caution. Until further information regarding these additional antigens has been obtained, the antibodies found in sera produced by the inoculation of LS fraction are to be preferred for the identification of the L and S antigens.

SUMMARY AND CONCLUSIONS

Observations on antibody production in the rabbit in response to the injection of the LS antigen of vaccinia have shown that this antigen retains its ability to stimulate the production of L and S antibodies, not only when it is in a state of solution as found in lapine Seitz filtrates, but also after it has been partially purified. As reported in previous papers, both the L and S antibodies participate (*a*) in the true agglutination of washed elementary bodies, (*b*) in the precipitation of the soluble LS antigen found in fresh vaccine suspensions or dissociated *in vitro* from washed elementary bodies. The difference in the thermostability of the L and S components of the LS antigen as far as their reactivity *in vitro* is concerned, holds in respect of their capac-

ity to stimulate antibody production. Heating at 70°C. invariably abolishes the antigenicity of the L component while the S component remains antigenic after being heated to 90°C.

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