IMMUNOLOGICAL STUDIES OF A HEAT-STABLE SUBSTANCE ISOLATED FROM TISSUES INFECTED WITH VACCINE VIRUS

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There are present in extracts of vaccine virus-infected tissues soluble substances which precipitate specifically when mixed under the proper conditions with antivaccinal immune serum. At least one of these is destroyed by boiling in aqueous solution and it has therefore been designated as the "L," or heat-labile substance. The other, resistant to boiling in neutral solution, is designated as the stable ("S") substance (1). Our recent studies have been concerned chiefly with the latter material and in a previous communication (2) we have reported the isolation from tissues infected with vaccine virus of a heat-stable, alcohol-soluble substance, presumably a pure protein, which precipitates specifically with antivaccinal serum. It was not determined whether this material was antigenic.

It has been stated that the heat-stable soluble specific substances of vaccine virus were not antigenic in rabbits. Smith (3) used a partially purified preparation, made by boiling an extract of virus-infected testicles at pH 5.5, 8.0, and 7.0 successively, and removing the coagulated protein at each step. He reported that the injection of this material into rabbits was followed by no detectable antibody response and suggested that in the manner of its action the substance resembled the bacterial haptenes. Ch'en (4) employed the technic of Smith as a preliminary step and then purified the active substance further by repeated precipitations with alcohol-ether and with alcohol and dialysis against tap water. He obtained a substance which he considers to be a polysaccharide and reported that it was non-antigenic in rabbits. On the other hand Craigie (5) has made preparations of the heat-stable substance which he partially purified by precipitation at pH 4.6. He states that these substances elicit a prompt antibody response when injected into rabbits which have previously been vaccinated. Since these animals have, or have once had, antibodies against the substance the response is a secondary one.

Craigie does not report on the response of normal rabbits to injection with his heat-stable substance.

The work of Craigie suggests that the heat-stable substance with which he is working is antigenic since it is capable of stimulating a prompt secondary formation of antibodies in already immune rabbits. But the failure of heated, serologically active extracts to elicit antibody formation in the experiments of Smith and those of Ch'en would seem to indicate that the substances remaining in solution were of the nature of bacterial haptenes. The difficulty may be more apparent than real for it is well known that appreciable quantities of an antigen are required to initiate the formation of antibodies, while much smaller amounts will suffice to produce a rapid rise of antibody titer in the serum of animals once immunized but in whose serum antibodies may be no longer detectable.

EXPERIMENTAL

Since we had been able to isolate from vaccine virus-infected tissues a heat-stable substance, presumably a protein, which was evidently specifically related to vaccinia, it became of importance to determine whether or not it was antigenic. In this communication we describe the response of rabbits to repeated injection with a purified preparation of the material, and report the results of experiments designed to determine the relation of this to other antigens of vaccine virus. Experiments are also described which were made in an effort to learn the relationship between antibodies against this substance and immunity to vaccinia.

Materials and Methods

1. Preparation of Heat-Stable Substance.—The heat-stable precipitating substance was prepared as described in our previous communication (2). The method may be summarized as follows: An aqueous extract of vaccine virus-infected tissue was prepared and passed through a Seitz filter; the filtrate was incubated at 37°C. for 5 days, then boiled for 5 minutes and the coagulum which formed was discarded. The precipitate from 25 per cent saturation with ammonium sulfate was discarded. The concentration of ammonium sulfate was increased to 50 per cent of saturation, and the resulting precipitate was collected and dissolved in buffer solution (pH 7.0). After dialysis for 2 days against running tap water the material was centrifuged and the sediment of water-insoluble material discarded.

The reaction of the solution was adjusted to pH 4.6 and the precipitate collected. This was dissolved in buffer solution at pH 7.0 and the active substance precipitated by addition of 9 volumes of neutral absolute alcohol. The precipitate was dissolved in water and the alcohol was removed by dialysis. For reasons of economy most of the preparations were made from extracts of dermal vaccine virus.

2. *Pure Anti-L Serum.*—Serum against the labile substances of vaccine virus was prepared from antivaccinal immune serum containing both L and S precipitins. Antibodies against the S substances were removed from the serum by absorption with S antigen.

3. *Titration of Antibody Content of Sera.*—The methods of conducting agglutination, precipitation, and complement fixation reactions have already been described (6). Neutralization tests were carried out as follows: Serial tenfold dilutions of virus (in the form of washed elementary body suspension) were prepared in Locke solution. To 0.3 cc. of each dilution an equal volume of the serum to be tested was added. After a brief incubation 0.25 cc. of each mixture was inoculated intradermally in a rabbit, and the animal observed daily for the appearance of lesions. In order to avoid possible individual variations in susceptibility to vaccinal infection between animals, all samples of serum from a single experimental animal were tested in one rabbit.

4. *Tests of Resistance to Vaccinal Infection.*—After completion of the series of inoculations of antigen and collection of serum, the animals used were tested for resistance to infection with vaccine virus. For this purpose they were inoculated intradermally with serial dilutions of vaccine virus in the form of washed elementary bodies of vaccinia.

5. *Housing of Animals.*—In order to guard against accidental infection with vaccinia of animals which were receiving injections of the pure S substance, arrangements were made to keep them in separate animal quarters. In this way they were segregated from animals infected with vaccinia and were cared for by attendants who had no contact with vaccine virus.

Determinations of Antigenicity in Rabbits

Since the heat-stable substance which we had prepared was derived from rabbits, it was deemed advisable to prepare antisera in the same animal species in order to avoid confusion due to non-specific reactions.

A solution of the pure substance was prepared and distributed in ampoules, which were hermetically sealed. These were submerged in boiling water for 90 minutes in order to sterilize the contents and to destroy any L antigen which might be present. 3 rabbits were given a series of intraperitoneal inoculations of 1, 2, 4, 4, and 4 cc. of the solution at weekly intervals (the solution had a precipitating titer of 1:320). 2 weeks after the last injection the rabbits were bled; the serum was separated and tested for the presence of antibodies.

The serum obtained from the rabbits following the series of injections contained antibodies directed specifically against the heat-stable substance. Inspection of Table I, in which the results of titrations are recorded, reveals that the sera precipitated in dilutions of 1:8 to 1:32 with extracts of dermal vaccine virus and agglutinated the washed elementary bodies of vaccinia in dilutions of 1:128 to 1:256. That the reactions did not involve products of bacterial contamination is demonstrated by the positive complement fixation reactions. The antigen employed in these tests was vaccine virus, cultivated in a chick embryo-Tyrode solution medium according to the method of Rivers.

TABLE I

Titration of Serum Obtained from Rabbits before and after Injection with S Substance

Reaction	Antigen	Serum					
		360		361		492	
		Before	After	Before	After	Before	After
Precipitation	Extract dermal vaccine virus	—	1:32	—	1:32	—	1:8
Agglutination	Washed elementary bodies	—	1:128	—	1:256	—	
Complement fixation	Cultured vaccine virus	—	1:16	—	1:8	—	

Absorption Experiments

That the antibodies engendered in response to injections of the stable antigen were specific for vaccine virus has been shown. That they were specifically directed against the stable antigen was shown as follows:

1. Absorption of Serum with Stable Antigen.—A portion of the serum was absorbed with a solution of the heat-stable antigen by the following technic. The ratio of optimal precipitation was determined according to the method of Dean and Webb. Serum and antigen were mixed in the indicated proportion, incubated at 50°C. for 1 hour, and centrifuged at 4000 R.P.M. for 1 hour in the angle centrifuge. The supernatant fluid was removed, and after remaining in the ice box overnight was centrifuged. The clear solution resulting failed to precipitate when mixed either with stable antigen or with a fresh filtrate containing both L and S antigens. The protocol of such an experiment is given in Table II.

This experiment shows that absorption of the serum with stable antigen was capable of removing all of the precipitins against S antigen,

and that precipitins against L antigen had not appeared in the serum of rabbits injected with S substance.

TABLE II

Absorption of Anti-S Antibodies from Serum of Animals Injected with S Substance

Serum	Antigen	Dilution of serum							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
361 untreated	Fresh extract of dermal virus containing L and S antigens	+	++	++	++	+	−	−	−
	Boiled extract of testicular virus containing S antigen	++	++	++	+	±	−	−	−
	Suspension of heated elementary bodies containing S antigen			±	+	+	±	±	−
361 absorbed with S antigen	Fresh extract of dermal virus containing L and S antigens	−	−	−	−	−	−	−	−
	Boiled extract of testicular virus containing S antigen	−	−	−	−	−	−	−	−
	Suspension of heated elementary bodies containing S antigen	−	−	−	−	−	−	−	−

TABLE III

Absorption of S Antigen from a Mixture of L and S

Antigen solution	Test serum	Antigen dilutions					
		1:2.5	1:5	1:10	1:20	1:40	1:80
Fresh extract of dermal virus containing L and S antigens	L serum*			++	++	+	−
	S serum†			++	++	−	−
	Normal serum			−	−	−	−
Fresh extract absorbed with anti-S serum	L serum	++++	++	++	+	−	−
	S serum	−	−	−	−	−	−
	Normal serum	−	−	−	−	−	−

* S antibodies removed by absorption.

† Prepared against pure S substance.

2. Absorption of Antigen with Pure Serum.—A filtrate of dermal vaccine virus was prepared which contained both L and S antigens. The optimal ratio was determined for precipitation with anti-S serum, and to a quantity of filtrate the calculated amount of serum was added. The mixture was incubated at 50°C. for

30 minutes, held in room temperature for 2 hours, and centrifuged at 4000 R.P.M. for 1 hour in the angle centrifuge. After remaining in the ice chest overnight, the solution was again centrifuged and the clear solution tested for the presence of the precipitating substance. The results are shown in Table III.

As is recorded in Table III, the crude skin filtrate which had been absorbed with our serum failed to yield a precipitate when more of the same serum was added. The addition of serum containing antibodies against the heat-labile substances gave a precipitate in almost as high dilutions of filtrate as before absorption. The anti-S serum had removed the heat-stable substances specifically, while leaving the heat-labile substances in solution.

TABLE IV

Neutralization Tests with Serum of Animals Injected with Pure S Substance

Serum	Highest dilution of virus causing lesion when mixed with undiluted serum
360 (anti-S)	10^{-4}, 10^{-4}
361 (anti-S)	10^{-4}, 10^{-4}
Normal control	10^{-5}, 10^{-6}
Immune control	$>10^{-1}$,* $>10^{-1}$

* No lesion produced by virus diluted 10^{-1} when mixed with immune serum.

Relation of Antibodies against the Heat-Stable Antigen to Vaccinal Immunity

Since it had been demonstrated that injection of the S antigen into rabbits caused antibodies against it to appear in the serum of these animals, it became of importance to determine whether this anti-S serum was capable of neutralizing the infective capacity of vaccine virus, and whether the animals themselves were resistant to inoculation with this pathogenic agent. This was determined in the following manner.

Neutralization tests were carried out as described above under Methods, as was also the determination of resistance of the rabbits to infection with vaccinia. The results of the neutralization tests are collected in Table IV.

Inspection of Table IV reveals the fact that the serum of the animals injected with S substance apparently neutralized small amounts of virus. The difference, however, in the effect on vaccine virus between the normal and anti-S sera is very slight and it may be

questioned whether it is sufficient to be taken as an indication of actual immunity. Furthermore, inoculation of minimal amounts of virus gave rise to infection in the tested animals. The lesions produced by a given dilution of virus appeared to be of the same severity in all the animals.

SUMMARY AND CONCLUSIONS

We have shown that it is possible to obtain from extracts of vaccine virus-infected tissues a substance or substances, apparently protein, which are serologically active, and which are specifically related to vaccinal infection. The present investigation is concerned with a study of their immunological reactions. Their intraperitoneal injection in rabbits is followed by the appearance in the serum of these animals of antibodies directed specifically against them. The precipitating capacities of this serum are entirely removed after addition of appropriate quantities of heated vaccine virus extract, indicating that antibodies against only the heat-stable antigens have been produced. Further evidence of the specificity of the antibodies is gained from the reverse experiment, that is, absorption of a virus extract with the serum. We have shown that under suitable conditions the serum will remove S antigen specifically leaving the labile substances in solution. This would apparently indicate that they are serologically distinct although both are vaccinal products.

Serum of animals injected with the S substance and containing antibodies against it in high titer is apparently capable of neutralizing minute amounts of active virus. The animals providing the serum are, however, without demonstrable resistance to vaccinia. The significance of the neutralizing activity of the serum is debatable because it is of a greatly different order of magnitude from that which follows infection with vaccinia.

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